GENETIC MANIPULATION OF CYTOKININ LEVELS IN NEW PLANT TYPE RICE

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Dedicated to my parents

DECLARATION

The research was carried out in Plant Molecular Biology Laboratory, International Rice Research Institute, Philippines, Institute of Experimental Botany, Prague, Czech Republic and the Norman Borlaug Institute for Plant Science Research, De Montfort University, Leicester, UK. This thesis is entirely my own work and has at no time been submitted for another degree.

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I certify that this statement is correct.

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CONTENTS

ABBREVIATIONS

bp	base pair
cDNA	copy DNA
CKs	cytokinins
c-Z	*cis* -zeatin
c-Z7G	*cis*-zeatin-7-glucoside
c-Z9G	*cis*- zeatin-9-glucoside
c-ZOG	*cis*- zeatin-*O*-glucoside
c-ZROG	*cis*- zeatin-riboside-*O*-glucoside
c-ZR	*cis*-zeatin-riboside
2,4-D	2,4-dichlorophenoxyacetic acid
DHZ	dihydrozeatin
DHZ7G	dihydrozeatin-7-glucoside
DHZ9G	dihydrozeatin-9-glucoside
DHZOG	dihydrozeatin-*O*-glucoside
DHZR	dihydrozeatin-riboside
DHZROG	dihydrozeatin-riboside-*O*-glucoside
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
E. coli	*Escherichia coli*
EDTA(Na_2)	Ethylenediamine tetra-acetic acid (disodium salt)
FW	fresh weight
g	gram
gus A	gene encoding ß-glucuronidase enzyme
l	litre
HCl	hydrochloric acid
HPLC/MS	high performance liquid chromatography/mass spectrometry
hpt	gene encoding hygromycin phosphotransferase
iP	N^6-(2-isopentenyl)adenine
iP7G	N^6-(2-isopentenyl)adenine –7-glucoside
iP9G	N^6-(2-isopentenyl)adenine–9-glucoside

iPR	N^6-(2-isopentenyl)adenine-riboside
ipt	gene encoding isopentenyl transferase
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pair
V	volts
LB	Lauria Bertani
M	Molar
min	minutes
ml	milliliter
mM	millimolar
mg	milligram
MS	Murashige and Skoog
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	nanogram
nos	nopaline synthase
PCR	polymerase chain reaction
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulphate
SSC	Sodium-saline citrate
TAE	Tris-acetate-EDTA buffer
Taq	*Thermus aquaticus*
T-DNA	Transferred DNA
TE	Tris-EDTA buffer
Tris	Tris-hydroxymethylaminomethane
T_0	the primary transformants
T_1	first generation of transgenic plants
T_2	second generation of transgenic plants
µg	microgram
µl	microliter

uv	ultraviolet light
v/v	volume/volume ratio
WAF	weeks after flowering
w/v	weight/volume ratio
X-gal	5-bromo-4-chloroindol-3-yl- β-galactopyranoside
X-gluc	5-bromo-4-chloro-3-indolyl- ß-D-glucuronide
Z	*trans*-zeatin
ZOG	*trans*-zeatin-*O*-glucoside
Z7G	*trans*-zeatin-7-glucoside
Z9G	*trans*-zeatin-9-glucoside
ZR	*trans*-zeatin-riboside
ZROG	*trans*-zeatin-riboside-*O*-glucoside

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ABSTRACT

The changes in chlorophyll content, rate of senescence and cytokinin (CK) levels in the top three leaves of field-grown New Plant Type (NPT) rice were determined during the grain filling period. The chlorophyll content in the leaves decreased from flowering to maturity. Fast-senescing and slow-senescing NPT rice lines were identified. The presence of 22 different CKs in the leaves of fast-senescing and slow-senescing NPT lines was reported for the first time. The 22 CKs were placed into four functionally different groups of CKs: active CKs (CK bases and ribosides), storage CKs (CK *O*-glucosides), inactive CKs (CK-7-*N*-glucosides and CK-9-*N*-glucosides), *cis*-zeatin and its derivative CKs. The results showed that it is not the actual content of CKs but the changes in dynamics of CKs which are decisive for fast senescence. The grain filling percentage was positively and significantly correlated with the rates of senescence in the top three leaves. The grain yield was significantly correlated with the rate of senescence only in the flag leaf which indicates the importance of the flag leaf in supplying the photoassimilates to the grains.

The isopentenyl transferase *(ipt)* gene, from *Agrobacterium-tumefaciens*, is involved in the first rate-limiting step in cytokinin biosynthesis. Using biolistic transformation, the *ipt* gene under the control of a senescence-specific SAG12 promoter (SAG12-*ipt*) was introduced into NPT rice to delay leaf senescence. The *ipt* gene was successfully integrated into the genome and expressed in the leaves of transgenic plants. The grain filling percentage was lower in the transgenic SAG12-*ipt* T_0 plants compared to the control plants. The levels of Z, Z9R, iP and iP9R in the leaves of plant No. T2068 and the levels of Z9R, DHZ9R and iP in the leaves of plant No. T1193 were higher than in the leaves of control plants. PCR analysis showed that the *ipt* gene was inherited in the T_1 progeny of the transgenic T_0 plants. The *ipt* gene was inherited in a 3:1 segregation ratio in the T_1 progeny of transgenic plant No. T1193 which indicates that the *ipt* gene was inherited as a single Mendelian locus. The grain filling percentage was lower in the *ipt*-positive T_1 plants of plant No. T1193 compared to the *ipt*-negative T_1 plants of plant No.

T1193 and the untransformed control plants. Four T_1 progeny of plant No. T1193 and their T_2 progeny showed delayed leaf senescence, shorter plant height, two to four week delay in flowering and lower grain filling compared to the control plants which might be due to overexpression of the cytokinins. The levels of Z9R and iP in the leaves of T1193-24-9 plant and the levels of Z9R, DHZ9R, iP and iP9R in the leaves of T1193-27-8 plant were higher than in the leaves of the control plant.

Since the *Arabidopsis thaliana SAG12* promoter did not seem to work well in the monocot background of rice, efforts were made to isolate the homologue of the *SAG12* promoter in NPT rice. The expression of *Arabidopsis SAG12* and senescence-related genes was examined in various parts of NPT rice. A phylogenetic tree showed that the *SAG12* gene had homology with several cysteine proteases in cereals, such as, maize, barley and rice and clustered closest to two rice BAC clones, namely, OSM146118 and OSM146316. However, the OSM146118 rice BAC clone was not senescence-specific. The OSM146118 BAC clone was expressed in equal intensity in the non-senescing, senescing and senesced leaves, hence, it is not senescence-specific. After RT-PCR analysis of the OSM146316 rice BAC clone, the transcripts found in non-senescing, senescing and senesced leaves were cloned and sequenced. The clones obtained in the senescing leaves showed homology with *Prunus armeniaca*'s ethylene-forming enzyme. Tblastn result of the ethylene-forming enzyme showed that it had homology with OSM13394 rice BAC clone. However, this rice BAC clone was expressed in equal intensity in all parts of the riceplant, hence, it is not senescence-specific. The expression of senescence-related genes, namely, *SAG101, ORE9, red chlorophyll catabolite, YLS3* and *chlorophyllase*, in NPT rice was determined. These senescence-related genes had some homology with several rice BAC clones, such as OSM14989, OSM1359, OSM151086, OSM1366 and OSM1282. RT-PCR analysis showed that these rice BAC clones were expressed in all or most parts of the rice plant, hence, none of them were found to be senescence-specific. In further studies, differential display or screening the cDNA library may be used to isolate the rice homologue of the SAG12 promoter.

Chapter 1. GENERAL INTRODUCTION

1.1 Importance of rice

Rice (*Oryza sativa*) is the world's most important cereal crop and a primary food source for more than a third of the world's population (Khush, 1997). More than 90% of the world's rice is grown and consumed in Asia where about two-third of the world's poor people live (Khush and Virk, 2000). Rice accounts for 35 to 60% of the calories consumed by 3 billion people in Asia (Khush, 1997). The population of rice consumers is increasing at the rate of 1.8% a year but the rate of growth in rice production has slowed down (Khush and Virk, 2000). To meet the projected demand in 2030, it is estimated that global rice production must reach 800 million tons of paddy rice which is 200 million tons more than the rice production in 2000 (FAOSTAT, 2003). This additional demand of rice needs to be produced in spite of the many constraints, such as less availability of land and water, less use of pesticides and less resources (Khush and Virk, 2002). There is rapid decline in the cultivated area due to urbanisation, infrastructure development and industrialisation (Peng *et al.,* 1999). Moreover, a decrease in rice production is a result of yield losses due to abiotic and biotic stress. Hence, we need to develop rice varieties with higher yield potential and better nutritional value using all the available conventional and non-conventional methods to meet the increased demand for rice production (Khush and Virk, 2000).

1.2 Methods for rice improvement

1.2.1 Conventional plant breeding for rice improvement

The conventional plant breeding method of selection and hybridization is a time-tested strategy for developing rice cultivars with higher yield potential (Khush and Virk, 2000). The tall conventional plant type of rice was hybridized with the

dwarf variety to produce semi-dwarf varieties. The improved high yielding varieties are a semi-dwarf plant type, with high tillering, sturdy stems and dark green erect leaves (Figure 1.1). Breeding of semi-dwarf rice varieties such as IR8 in the 1960s and 1970s resulted in quantum leaps of yield potential which marked the green revolution in Asia (Peng and Khush, 2003). The modification in plant architecture helped to double the yield potential of rice (Virk and Khush, 2002). World rice production doubled from 257 million tons in 1966 to 520 million tons in 1990 (Khush, 1995). About 74% of the rice land in Asia is currently planted with these improved semi-dwarf varieties. However, there has been no major increase in the maximum genetic potential of the high-yielding semi-dwarf rice varieties since the 1960s (Borlaug, 2002). The yield of IR8 is between 9 to 10 tons/ha under favourable irrigated conditions at the International Rice Research Institute (IRRI) in the Philippines. The yield of the recently bred semi-dwarf rice cultivars such as IR72 is also between 9 to 10 tons/ha. This result indicates that the yield potential of irrigated rice varieties has stagnated for the past 30 years, even when rice is grown in the most favourable conditions (Peng *et al.*, 1994). The large number of unproductive tillers, limited sink size and lodging susceptibility were identified as the major constraints to increased yield potential in these semi-dwarf rice cultivars (Peng *et al.*, 1999).

To increase the yield potential of rice beyond 10 t/ha, various strategies are being utilized such as ideotype breeding, wide hybridization, heterosis breeding and genetic engineering (Khush and Virk, 2000). In ideotype breeding, a plant type is defined which is theoretically more efficient than existing cultivars based on the knowledge of physiology and morphology (Peng *et al.*, 1999). Plant breeders then started to select for the ideotype with the desired characteristics. In 1989, modifications to the plant architecture of the high-yielding rice varieties were proposed in IRRI to create the New Plant Type (NPT) rice (Figure 1.1; Khush, 1995; Peng *et al.*, 1994; Virk and Khush, 2002) with the following characteristics: 200-250 grains per panicle, 90-100 cm tall, lower number of tillers (8-10 tillers when transplanted), no unproductive tillers, very sturdy stems,

Figure 1.1 Sketches of different plant types of rice. (A) Tall conventional type, (B) Improved high yielding, high tillering and semi-dwarf plant type, and (C) Proposed low tillering ideotype with higher harvest index and higher yield potential (Adapted from Khush, 1995).

dark green thick and erect leaves, vigorous root system, 100-130 days growth duration, multiple disease and insect resistance and acceptable grain quality.

1.2.2 Biotechnology for rice improvement

Biotechnology is another important tool for achieving improved rice varieties with higher yield potential, superior grain quality, enhanced resistance to pests and diseases and greater tolerance for stresses such as drought, cold and nutrient deficiencies (Maclean *et al.*, 2002). The techniques used in rice biotechnology include embryo rescue, anther culture, molecular marker-aided selection, DNA fingerprinting and transformation (Table 1.1). These techniques allow the plant breeders to accomplish objectives that cannot be achieved through selection and hybridization.

Table 1.1. Applications of biotechnology techniques to rice improvement (adapted from Maclean *et al.*, 2002).

Technique	Application
Embryo rescue	Transfer of genes from wild rice to cultivated rice
Anther culture	Rapid stabilisation of new lines
Molecular marker-assisted selection	Acceleration of breeding programs by use of genetic markers rather than phenotypic selection
DNA fingerprinting	Identification of genetic variation for resistance against pests and pathogens
Transformation (*Agrobacterium* and biolistic methods)	Introduction of novel genes into rice for tolerance to abiotic stress (such as drought or salinity), resistance against pests and diseases, improvement of nutritional quality and enhancement of yield

1.2.2.1. Embryo rescue

The genus *Oryza,* to which cultivated rice (*Oryza sativa* L.) belongs, has more than 20 wild species. These wild species are an important reservoir of useful genes for resistance to major diseases and insect pests and tolerance to abiotic stresses. To transfer the desired trait, the wild species are hybridized/crossed with cultivated rice. However, very low seed set is obtained and few hybrid seeds are poorly developed due to cross incompatibility between the hybridized parents. Most of the hybrid embryos are generally weak and start degenerating two weeks after pollination because of the incompatibility between the genomes. To overcome the problem of degenerating hybrid embryos, embryo rescue is utilized (Hu and Wang, 1986).

Embryo rescue is a technique wherein hybrid embryos are excised and grown on a simple inorganic medium with a supplemental energy source for 10 days to produce hybrid seedlings (Jena and Khush, 1984). After 14 days of pollination between wild species and cultivated rice, immature hybrid embryos are taken and surface sterilized in sodium hypochlorite solution (35%) supplemented with 2 drops of Tween-20. After washing the embryos in sterilized water, the delicate young embryos are excised and asceptically isolated under a stereomicroscope in laminar flow. The isolated embryos are cultured asceptically on 1/4 Murashige and Skoog's (MS) medium and incubated in the dark at 25 degrees celsius until germination. The seedlings, growing *in vitro*, are kept in a lighted incubation room until the seedlings have formed three leaves before the seedlings were transferred to soil. Embryo rescue is important for overcoming seed dormancy and seed sterility, *in vitro* regeneration and rescuing incompatible hybrid crosses.

1.2.2.2. Anther culture

The finding that haploid rice plants can be produced by anther culture was important to development of rice cultivars (Niizeki and Oono, 1968). Anther culture involves the *in vitro* culture of anthers containing pollens. The immature pollen either gives rise directly to embryos or to callus tissue. The callus tissue is induced to regenerate plants under the influence of growth regulators added to the culture medium. Pollen is haploid and the cells produced from pollen during culture are haploid as well. When plants are regenerated from haploid cells, a haploid plant is produced. Haploid plants are sterile and can produce no seed. However, a spontaneous duplication of chromosomes often occurs within anther culture-derived callus cells which results in the production of fertile double haploid plants. Because the two copies of genetic information within such plants are identical, the plants are fully homozygous plants.

Anther culture enabled the rapid creation of homozygous lines, thus, reducing the time required for breeding new cultivars by at least 3 to 5 years (Morrison and Evans, 1988). The use of double haploids for breeding also improves the efficiency with which superior genotypes can be identified (Snape, 1989). Aside from serving as a tool for cultivar development, populations derived by anther culture have enabled researchers to map molecular markers efficiently and to characterize genetically complex traits.

1.2.2.3 Molecular marker-assisted selection

The use of DNA molecular markers in marker-assisted selection to aid conventional plant breeding is now well established. The genetic marker may be a recognizable phenotypic trait (eg. height, colour, response to pathogens) or a molecular trait (eg. DNA molecular marker). Phenotypic markers depend on expression of genes and are limited to those genes expressed at a particular time or under particular developmental or environmental conditions. But DNA molecular markers provide an almost unlimited supply of markers that identify

specific sequences across the genome. The molecular markers are independent of developmental stage, environment or gene expression (Jones, 2002).

Molecular markers are used for genetic mapping of many genes involved in simple and complex traits of rice (Nagamura *et al.*, 1997). The first molecular genetic linkage map was derived using restriction fragment length polymorphism (RFLP) markers (McCouch *et al.*, 1988). But the expensive cumbersome nature of hybridization-based RFLP analysis limited their use in large-scale breeding efforts. The generation of RFLP data is time-consuming and costly to perform as many steps are involved, usually requiring either radioactive or non-radioactive detection method to identify the polymorphic bands. Large quantities of DNA (generally 5 to 10 μg of DNA) are required and the generation of RFLPs is technically demanding. But polymerase chain reaction (PCR)-based markers have a number of advantages over RFLP markers. Using PCR-based markers, low amounts of DNA (5-50 ng of DNA) are required which is 1000-fold less than the amount required using RFLP markers. Hence, the development of PCR-based markers has greatly reduced the cost and efficiency of using DNA markers to tag genes (McCouch *et al.*, 1997).

Many markers have been identified which are closely linked to genes for agronomic traits of interest such as genes for developmental traits, quality traits, resistance to pests and diseases and tolerance to abiotic stresses. The PCR-based markers, such as, randomly amplified DNA (RAPD) markers (Redona and MacKill, 1996), amplified length polymorphism (AFLP) markers (Maheswaran *et al.*, 1997) and simple sequence repeat (SSR) markers (McCouch *et al.*, 1997), are now widely used in various rice breeding programs around the world (Chen *et al.*, 2000).

Molecular marker-assisted selection for rice improvement allows increasing speed and accuracy of selection, stacking of genes, following genes in backcross populations and reduced costs of field-based selection. Hence, instead instead of growing breeding lines in the rice field and challenging or testing for important

traits over the growing season, it is possible to extract DNA from seedlings and use the molecular markers to detect the presence of the desired trait in one day. Plants which do not have the genes for the desired trait can then be removed early in the breeding programme. Hence, marker aided selection is a highly cost-effective and efficient method for rapid selection of agronomic traits.

1.2.2.4. DNA fingerprinting

Molecular tools are extremely useful in the management and conservation of germplasm collections. DNA fingerprint data can be applied to determine the genetic diversity. DNA markers have been used to identify the wild progenitors of diverse species. The availability of PCR-based markers, such as SSRs, offers the opportunity for more fine-scale genetic characterization of germplasm collections than previously possible. SSR markers have high levels of polymorphism, occur throughout the genome and are easily detected. Furthermore, SSR markers have the additional advantage that many of the complications of environmental effects acting upon characters is avoided by looking directly at variation controlled at the genetic level (Godwin, 2003). SSR markers are increasingly used for investigations of critical importance to curators of germplasm collections including:

i. The establishment of unique genetic identities or fingerprints.

ii. The assessment of genetic diversity contained within a collection.

iii. The determination of genetic relatedness between accessions.

iv. The evaluation of core collections, wherein a core collection of plant genetic resources consists of a limited set of accessions chosen to represent the genetic variation in a crop species and its wild relatives.

1.2.2.5. Transformation

Among the techniques used in plant biotechnology, the most novel contribution is in the addition of foreign genes to the rice gene pool through transformation. Transformation allows the introduction of rice genes or genes from bacteria, viruses and other crops which are isolated and modified to give improved properties. Transformation of rice gives access to a broader gene pool and thus expands the range of gene transfer compared to that which was obtainable by conventional plant breeding.

1.2.3 Rice transformation

Rice transformation is done using techniques in plant tissue culture, microbiology and molecular biology. In the transformation process, plasmid DNA constructs containing a number of foreign genes (also called transgenes) are introduced into embryogenic cells which develop into transgenic plants under *in vitro* conditions. The foreign gene is a DNA segment or sequence of bases along a DNA molecule which comes from any source, such as, microbes, viruses, other plants or other rice varieties. Transformation is a powerful tool for genetic manipulation which achieves in a quick and precise way what the rice breeders try to accomplish by years of backcrossing. The introduction and expression of specific genes in plants provide a powerful tool for rice improvement. Rice transformation is being used to generate plants possessing traits unachievable by conventional plant breeding especially in cases where there is no source of the desired trait in the gene pool (Datta, 1999).

Improvements in the technology for the transformation of rice have been remarkable in the past few years (Hiei *et al.*, 1997). Various methods in tissue culture are available which can prepare the plant cells or tissues which will receive the gene of interest. Once the plant cells or tissues are prepared and ready, the foreign gene can be introduced using any of the various methods for rice transformation.

1.2.3.1 Tissue culture methods

The two major steps for rice *in vitro* culture are callus induction and plant regeneration. Once small pieces of tissue from any part of the plant (called explants) are placed in sterile culture on a solid nutrient medium, a callus forms. When auxin and cytokinin are present in the correct amount, somatic embryos are formed and then plantlets are regenerated. These two steps are genetically controlled but growth *in vitro* can be enhanced by choosing the best basal medium, right amount and combination of different hormones, amino acids and vitamins. Choosing the explant and medium for induction of callus is a vital step in rice *in vitro* culture.

a. Induction of callus

Rice callus induction is affected by genotype, medium and genotype-medium interaction. There are different responses of the genotype to the callus induction medium. The most commonly used callus induction media solidified with agarose are the following: MS (Murashige and Skoog, 1962) and N6 (Chu *et al.*, 1975).

The most commonly used explants for callus induction are immature embryos, anther and scutella in mature seeds. Callus formation is induced by higher concentrations of auxin than cytokinin. The most commonly used auxin is the synthetic 2,4-D. The callus induction efficiency is evaluated by counting the number of embryogenic calli produced. Only the embryogenic calli have the capability to regenerate plants.

b. Plant regeneration

Rice plants can be regenerated from embryogenic calli. The semi-solid media used for plant regeneration have higher ratios of cytokinin to auxin. Plant regeneration requires light. The plant regeneration is genetically controlled and efficiency can be enhanced by choosing the best hormone ratios and gelling agent in the media.

1.2.3.2 Plasmid DNA used as cloning vectors for transformation

A plasmid is the extrachromosomal element of DNA found inside many bacteria. Plasmid is relatively small, covalently closed circular molecule which carries genes for antibiotic resistance or the metabolism of substrates. In the early 1970s, a number of naturally occurring plasmids were artificially modified and constructed as cloning vectors.

One of the most notable plasmids, pBR322, was widely used because of the following desirable features:

i. The plasmid is smaller than a natural plasmid which makes it more resistant to damage by shearing and increases the efficiency of uptake by bacteria during transformation.

ii. A bacterial origin of DNA replication ensures that the plasmid will be replicated by the host cell.

iii. Two genes coding for resistance to antibiotics have been introduced. One of these resistance genes allows the selection of cells which contain the plasmid. Hence, only those cells which contain the plasmid will grow to form colonies when cells are plated on a medium containing an appropriate antiobiotic. The other resistance gene can be used for detection of those plasmids which contain the inserted DNA.

iv. There are single restriction sites for a number of restriction enzymes at various points around the plasmid which can be used to open and linearize the plasmid and insert the fragment of DNA into the plasmid.

The valuable features of pBR322 have been enhanced by the construction of a series of plasmids, termed pUC (produced at the University of California) which contain an antibiotic resistance gene for ampicillin and origin of replication for *E. coli*. Furthermore, the most popular restriction sites are concentrated into a region termed as multiple cloning sites. When the pUC plasmid has been utilized to transform the *E. coli* host cell, the gene may be switched on by adding the inducer called isopropyl-β-D-thiogalactopyranoside (IPTG). IPTG causes the production of β-galactosidase which is able to hydrolyse a colourless substance called 5-bromo-4-chloroindol-3-yl-β-galactopyranoside (X-gal) into a blue insoluble material. Hence, a recombinant pUC plasmid may be easily detected since it is white or colourless in the presence of X-gal whereas an intact non-recombinant pUC plasmid will be blue because its gene is fully functional and not disrupted. This blue/white selection allows the initial identification of recombinants and has been included in a number of subsequent vector systems (Raphley, 2002).

1.2.3.3 Rice transformation methods

All transgenic rice research depends on the availability of procedures for plant transformation. There are two major methods used for plant transformation, namely, indirect and direct method of DNA transfer (Datta, 1999). *Agrobacterium tumefaciens*-mediated transformation, an indirect method of gene transfer, involves placing the foreign DNA into a vector or carrier before the DNA is transferred to a recipient plant cell or tissue. PEG-mediated, protoplast and biolistic transformations are direct methods of transformation which involve the introduction of the foreign DNA into the recipient cells or tissues of the plant using physical, electrical or chemical means. The different transformation methods used for introducing genes into rice are shown in Table 1.2. Among the different methods available for the gene transfer and successive regeneration of transgenic rice plants, the most commonly used methods are *Agrobacterium*-mediated transformation and biolistic transformation.

Table 1.2. Different methods used in rice transformation

Method of transformation	References
Agrobacterium-mediated transformation	Hiei *et al.*, 1994; Aldemita and Hodges, 1996; Zhang *et al.*, 1997
PEG-mediated transformation	Zhang and Wu, 1988; Datta *et al.*, 1990b; Hayashimoto *et al.*, 1990; Li *et al.*, 1991
Protoplast transformation	Toriyama *et al.*, 1988; Zhang *et al.*, 1988; Shimamoto *et al.*, 1989; Tada *et al.*, 1991
Biolistic transformation	Christou *et al.*, 1991; Datta *et al.*, 2003

a. *Agrobacterium*-mediated transformation

Agrobacterium-mediated transformation is a natural system of gene transfer from the bacterium to dicotyledonous plants. The virulent strains of *Agrobacterium* carries a tumor-inducing (Ti) plasmid. The transfer DNA (T-DNA), a small portion of the Ti plasmid, contain cytokinin, auxin and opine genes. During infection by *Agrobacterium tumefaciens,* only the T-DNA is incorporated into the nuclear DNA of wounded host plant cells. There are excellent reviews which document the development of *Agrobacterium*-mediated transformation (Gelvin, 2000; Zupan *et al.*, 2000).

A considerable number of plant transformation vectors for *Agrobacterium*-mediated transformation has been developed (Puddephat, 2003). The T-DNA of *Agrobacterium tumefaciens* is disarmed, by removal of cytokinin, auxin and opine genes, and replaced with the foreign gene of interest. *Agrobacterium* Ti-based vectors contain the following features:

i. the T-DNA region which is defined by the right and left border sequences

ii. the 35 bacterial virulence genes

iii. the multiple cloning sites for introducing target genes within the T-DNA region

iv. a bacterially expressed selectable marker gene which encode resistance to kanamycin, ampicillin, tetracycline or spectinomycin

The vectors used for *Agrobacterium*-mediated transformation are either integrative (or integrative) or binary (or autonomous) vectors (White, 1993). The integrative vectors are designed such that they cannot replicate in *Agrobacterium* without first integrating into the resident Ti plasmid whereas binary vectors are stably maintained in *Agrobacterium.* In the binary vector-containing strains of *Agrobacterium*, the genes required for infection by *Agrobacterium tumefaciens* and mobilization of T-DNA are contained in one plasmid that cannot integrate its DNA into the plant genome. The T-DNA sequences needed for integration of foreign DNAs into the chromosomes of plants are present on a second plasmid, hence it is called binary vector. The binary vector is utilized to carry the foreign DNA sequence but does not contain Ti genes. The availability of binary vectors containing selectable marker genes has greatly facilitated cloning and the introduction of foreign DNA into plant cells.

The previous notion that cereals could not be infected by *Agrobacterium* has been proven incorrect when Hiei *et al.,* (1994) provided unequivocal evidence for the successful production of japonica rice plants expressing hygromycin phosphotransferase (*hpt*) and β-glucuronidase (*gusA*) genes. There have been many reports on the successful transformation of cereals using *Agrobacterium*-mediated transformation (Hiei *et al.,* 1994; Hiei*et al.,* 1997; Aldemita and Hodges, 1996; Zhang *et al.,* 1997). Now both japonica and elite indica rice varieties were efficiently transformed using this method (Hiei *et al.,* 1994; Aldemita and Hodges, 1996; Zhang *et al.,* 1997).

Although there are some limitations associated with the use of *Agrobacterium*-mediated transformation such as genotype recalcitrance, unwanted vector backbone transfer, T-DNA repeats and minor rearrangements, it still offers some advantages over the biolistic method (Vain *et al.*, 2003). *Agrobacterium*-mediated transformation generally produces a higher frequency of single-copy transgenic plants which are important for many molecular genetic studies and crop improvement strategies. Komari *et al.*, (1996) reported that this method offers the possibility of obtaining unlinked co-transformed transgenes which permits the production of marker-free transgenic plants.

b. Biolistic transformation

Biolistic transformation is sometimes referred to as biological ballistics (biolistics), biolistic particle delivery system, microprojectile bombardment, gene gun or particle bombardment. Biolistic transformation is a process by which the microprojectiles carrying the foreign DNA or other biological materials are delivered into the plant cells.

John Sanford developed a microprojectile gun or gene gun to directly shoot DNA into plant cells (Sanford *et al.*, 1987). Since the first device reported by Sanford in 1987, various instruments have been developed for accelerating the microprojectiles (Vain *et al.*, 1995). The microprojectiles used are either gold particles (0.95 μm in diameter) or tungsten particles (0.7 μm in diameter) which can carry the DNA containing the gene(s) of interest. Gold is biologically inert whereas tungsten degrades DNA over time and can be toxic to some cell types, hence, the non-toxic gold particles is often used instead of tungsten particles (Russel *et al.*, 1993a). The high velocity required to penetrate the plant cell walls, is brought about by gunpowder, gas, air pressure or by electrical discharge (Sanford *et al.*, 1987). Those plant cells which incorporate the foreign DNA, containing the selectable marker gene, into their genome can survive the selection and give rise to transgenic plants.

Physical, environmental and biological factors affect the efficiency of biolistic transformation (Christou, 1992). There are pronounced effects brought by the nature, chemical and physical properties of the metal particles, nature, preparation and binding of DNA onto the particle (Christou, 1992). In early biolistic apparatus such as the original gun powder version, the bombardment or blast is a major cause of injury to the target cells which often inhibits regeneration of putatively transformed cells and subsequently reduces the efficiency of this technique (Russell *et al.,* 1993a; Christou, 1997). The commercial availability of biolistic equipments, such as the BioRad PDS 1000/He device, have facilitated the standardization of gene delivery parameters in many laboratories. Recently, Sudhakar *et al.,* (1998) used a portable, inexpensive helium-driven particle bombardment device and have shown transformation rates which are comparable to more sophisticated devices. The use of this instrument is beneficial for laboratories which are not able to purchase more expensive instruments for biolistic transformation.

Biolistic transformation is the most widely used plant transformation method today compared to *Agrobacterium*-mediated transformation and has been applied to a broad range of species of dicots and monocots. Luthra *et al.,* (1997) provided a list of dicot and monocot plants transformed, from 1987 to 1995, using the particle bombardment. Some of the dicot and monocot plants which were successfully transformed using biolistic transformation are shown in Table 1.3.

Biolistic transformation appears to be effective for all plant species tested and may be a "universal" gene delivery mechanism (Sanford *et al.,* 1987). This technique is genotype-independent, hence, it offers major advantages for transforming intact cells or tissues (Cao *et al.,* 1991) and recalcitrant species (Christou, 1992). More than 40 rice cultivars were reported to be transformed using biolistic method which shows that this method is genotype-independent (Datta *et al.,* 1998; Pinto *et al.,* 1999). The physical nature of the biolistic transformation, brought about by the use of high velocity microprojectiles, gives it the ability to deliver foreign DNA into individual regenerable cells, tissues or

Table 1.3. The different dicot and monocot plants successfully transformed using biolistic transformation

Type of plant	Plant	Reference
dicot	soybean	Christou *et al.*, 1988; McCabe *et al.*, 1988; Wang *et al.*, 1988; Christou *et al.*, 1991
	bean	Russel *et al.*, 1993b
	tobacco	Klein *et al.*, 1988c; Twell *et al.*, 1989
	cotton	McCabe and Martinell, 1993
monocot	oats	Somers *et al.*, 1992
	sorghum	Hagio *et al.*, 1991; Casas *et al.*, 1993
	tulip	Wilmink *et al.*, 1992
	onion	Klein *et al.*, 1988c
	maize	Klein *et al.*, 1988 a, b; Finer *et al.*, 1992; Gordon-Kamm *et al.*, 1990; Fromm *et al.*, 1990; Koziel *et al.*, 1993; Vain *et al.*, 1993
	sugar cane	Bower and Birch, 1992
	rye	Castillo et al., 1994
	wheat	Wang *et al.*, 1988; Lonsdale *et al.*, 1990; Vasil *et al.*, 1990; Vasil *et al.*, 1992; Weeks *et al.*, 1993; Becker *et al.*, 1994; Nehra *et al.*, 1994
	barley	Jahne *et al.*, 1994
	rice	Christou *et al.*, 1991; Cao *et al.*, 1992; Li *et al.*, 1993; Ghosh Biswas *et al.*, 1998

organs. There is no biological limitation to the actual process of DNA delivery, hence, genotype is not a limiting factor and it is genotype-independent.

The advantages of using microprojectile bombardment are the following: a.) it is cultivar-independent and species-independent, b.) it is simple to perform, and c.) it bypasses any host specificity associated with *Agrobacterium*-mediated transformation (Davey *et al.*, 2000). Biolistic transformation offers major advantages for transformation of intact plant cells or tissues (Cao *et al.*, 1991; Finer *et al.*, 1992), rapid recovery of transformed T_1 seeds and transformation of recalcitrant species (Christou, 1992). Several commercially important rice cultivars such as Gulfmont, Lemont and Indica cultivars were transformed using biolistic transformation. Kim (1993) obtained stable integration of β-glucuronidase (*gusA*) and hygromycin phosphotransferase (*hpt*) genes in the Korean cultivar, Anjungbyeo, using the biolistic method. Ghareyazie *et al.*, (1997) obtained transgenic plants from Iranian aromatic rice varieties such as Tarom Molai and Khazar containing *hpt* and *Bacillus thuringensis* (*Bt*) genes. Datta *et al.*, (2003) produced many transgenic plants from different rice cultivars which express the phytoene synthase (*psy*) gene and enhanced which vitamin A in the rice grains.

Biolistic transformation is widely used because it circumvents two major limitations of the *Agrobacterium*-mediated transformation. First, the use of biolistic transformation allows the introduction of transgenes into any type of cells or tissues and in any plant genotype because the delivery of DNA is controlled entirely by physical instead of biological parameters. Second, biolistic transformation permits the stable and heritable introduction of many different genes at once using different plasmids as these tend to concatemerize to form one DNA cluster that integrates at a single locus (Twyman *et al.*, 2002). After cotransformation of rice with 14 separate plasmids containing various marker genes, molecular analysis showed the cointegration of 13 of the plasmids in one

plant (Chen *et al.*, 1998). Four agronomically-important genes expressed in rice plants (Tang *et al.*, 1999).

Clean DNA and agrolistic systems are the recent advances in the use of particle bombardment technology. These two systems limit the amount of plasmid backbone sequence which enters the plant genome because the transgene integration may be facilitated by damage caused to DNA strands as the particle moves through the nucleus. The exogenous DNA sometimes integrates at two different sites separated by megabasepairs of DNA although these still segregate as a single locus (Twyman *et al.*, 2002). Recently, the use of linear transgene constructs lacking vector backbone sequences in biolistics has been shown to produce transgenic plants with low-copy number and simple integrations of genes (Fu *et al.*, 2000).

One potential disadvantage of the particle bombardment method is the high cost of purchasing a bombardment device. Another disadvantage is the tendency for the introduced DNA sequences to undergo complex rearrangements before or during integration. The forces involved in the particle bombardment may cause more DNA fragmentation compared to other direct methods of DNA delivery (Twyman *et al.*, 2002).

1.2.3.4 Promoters and genes used in transformation

a. Promoters used in transformation

Promoters have been used to control the expression of selectable marker, reporter and agronomically-important genes in rice (Li *et al.*, 1997). The three types of promoters are constitutive, inducible and tissue-specific promoters.

i. Constitutive promoters

A constitutive promoter is active in all tissues and developmental stages of the plant. The most commonly used constitutive promoters are cauliflower mosaic virus (CaMV 35S), maize ubiquitin (Ubi1), maize (Adh1) and rice actin (Act1) promoters (Table 1.4). CaMV 35S promoter has been the most commonly used constitutive promoter (Benfey and Chua, 1990). The CaMV 35S promoter is reported to work well in many dicotyledons but this promoter is less effective in monocotyledons (Christensen *et al.*, 1992). It is inactive in pollen of rice (Bruce *et al.*, 1989). However, the CaMV 35S promoter gives low level expression in transformed rice cells (Cao *et al.*, 1992). The high activity of Ubi1 promoter has been transiently or stably observed in rice (Bruce *et al.*, 1989). The rice Act1 5' region is an efficient promoter for driving high level expression of foreign genes in transgenic rice (Mc Elroy *et al.*, 1990). The rice Act1 promoter was 5-10 times more active than maize Adh1 promoter transformed rice cells (Zhang *et al.*, 1991). In many cases, constitutive promoters are used to transcribe the genes of interest. However, a major limitation of constitutive promoters is that they cannot be used to investigate genes whose constant over-expression or under-expression has deleterious effects on the plant (Zuo *et al.*, 2001). This limitation can be overcome by using inducible or tissue-specific promoters.

ii. Tissue-specific promoters

The tissue-specific promoters allow the expression of the gene only in certain tissues, organs or cell types in the plants. The tissue-specific promoters used in rice is shown in Table 1.5. The advantage of using a tissue-specific promoter is that it drives the expression of the foreign gene in a specific tissue of the plant. For example, the *cryIA* gene from *Bacillus thuringensis* (*Bt*) confers resistance against the stem borers which are insect pests of rice that can cause yield losses. These stem borers feed on the leaves of young plants and then later on feed on the stem which damages the neck of panicle during the reproductive stage of rice.

Table 1.4. Constitutive promoters used in rice transformation (adapted from Datta, 1999)

Constitutive Promoter	Source	Reference
1. CaMV 35S	Cauliflower mosaic virus (CaMV)	Terada and Shimamoto, 1990; Datta *et al.*, 1990a; Battraw and Hall, 1990; Mitsuhara *et al.*, 1996
2. Emu	maize	Chamberlain *et al.*, 1994
3. Ubiquitin	maize	Toki *et al.*, 1992; Cornejo *et al.*, 1993
4. Actin1	rice	McElroy *et al.*, 1990; Zhang *et al.*, 1991
5. Adh1	maize	Kyozuka *et al.*, 1991

Table 1.5. Tissue-specific promoters used in rice transformation (adapted from Datta, 1999)

Tissue-specific Promoter	Source	Plant tissue	Reference
1. PEP	Maize	green tissue	Matsuoka *et al.*, 1994; Datta *et al.*, 1996
2. pith	Maize	pith tissue	Datta *et al.*, 1998
3. rolC	R1 plasmid	vascular and embryogenic tissue	Matsuki *et al.*, 1989
4. LHCP	Rice	leaves, stems and floral tissue	Terada *et al.*, 1993
6.Rbc-S	Rice	mesophyll	Kyozuka *et al.*, 1993
7.RTBV	RTBV	phloem tissue	Yin and Beachy, 1995
8.Osg 6B	Rice	tapetum	Yokoi *et al.*, 1997

To address this problem with stem borers, the *cryIA* gene is fused with the PEP-carboxylase promoter or pith-specific promoter. The use of PEP-carboxylase promoter, driving the expression of the *cryIA* gene in the green tissues, enhanced resistance against the stem borers (Ghareyazie *et al.,* 1997). Western blot analysis showed that the BT protein was present in the leaves but not in the mature grains of rice. The use of pith-specific promoter, driving the expression of the *cryIA* gene in the pith of rice, also enhanced the resistance against the stem borers (Tu *et al.,* 2000).

Another example of the advantage of using tissue-specific promoter is the use of seed-specific promoter to enhance the nutrition quality of rice grains. Seed-specific glutelin promoter was used to drive the expression of *psy* gene (Datta *et al.*, 2003) or *ferritin* gene (Vasconcelos *et al.,* 2003). Transgenic rice plants were produced wherein the endosperm is yellow because of the accumulation of carotenoid compounds in the endosperm (Datta *et al.,* 2003). Vasconcelos *et al.,* (2003) reported that the expression of the *ferritin* gene leads to higher iron and zinc levels in the transgenic indica rice grains.

iii. Inducible promoters

The inducible promoters trigger the expression of the gene only when stimulated by specific induction agents such as wounding (Keil *et al,* 1989), heat shock (Prandhl and Scoffl, 1996), light (Kuhlemeir *et al.,* 1987) or pathogens (Guerineau, 1995). The isopentenyl transferase (*ipt*) gene was fused to various types of inducible promoters such as light-inducible promoter (Hamdi *et al.,* 1995; Garnier *et al.,* 1996), wound-inducible proteinase-inhibitor-IIK (*pinII*) gene from potato (Smigocki, 1995), copper (Cu)-inducible promoter (Mc Kenzie *et al.,* 1998), soybean heat shock promoter HS6871 (Smart *et al.,* 1991) and heat-inducible hsp70-promoter from *Drosophila melanogaster* (Van Loven *et al.,* 1993; Rupp *et al.,* 1999).

To achieve a more effective regulation of transgene expression, a series of chemically regulated expression systems have been developed wherein expression can be switched on or off by adding or removing specific chemicals, the inducer or inhibitor (Wang *et al.,* 2003). In the activation of chemical-inducible expression systems, dexamethasone (dex) and ethanol are inducers of gene expression. In transgenic tobacco plants, the expression of a luciferase (*luc*) reporter gene driven by the target promoter is stimulated over 100-fold by treatment with dex, a synthetic ligand (Aoyama and Chua, 1997). The AlcR activator, from *Aspergillus nidulans*, was used to control the expression of the target genes in plants using ethanol. In transgenic tobacco plants, AlcR stimulated the expression of a chloramphenicol acetyltransferase (*cat*) reporter gene upon induction by ethanol to a level corresponding to 50% activity of the CaMV 35S promoter, whereas the background was nearly detectable (Caddick *et al.,* 1998).

Roslan *et al.,* (2001) has characterized the ethanol-inducible alc gene-expression in *Arabidopsis thaliana* by linking the alc promoter to *gusA, luc* and *gfp* genes. Direct application of ethanol or exposure of the whole plants to ethanol vapour are equally effective means of induction. On application of the inducer, alc-mediated expression was rapid and detected within one hour. Maximal expression using soil-grown plants occurred after 5 days of induction but there is neglible activity in the absence of exogenous inducer. The alc gene-expression system has potential for use in agriculture.

b. Genes used in rice transformation

There are three types of genes, which are commonly used in transformation, namely, the selectable marker genes, reporter genes, and agronomically-important genes.

i. Selectable marker genes

After using any of the transformation methods, only a few cells stably integrate the DNA into their genome and become transformed (Franks and Birch, 1991). Most of the cells which remain untransformed must not be allowed to grow into whole plants and must be killed using herbicide or antibiotic. It is vital to select the transformed cells amidst the large number of untransformed cells (Birch and Bower, 1994). To achieve this selection of transformed cells, a selectable marker gene is introduced into the plant together with the gene of interest.

Since the transformed and untransformed cells do not differ in appearance, the use of a selectable marker gene is important in selecting the transformed cells (Birch and Bower, 1994). Selectable marker genes, conferring resistance to herbicides or antibiotics, are used to distinguish the transformed plant cells from the untransformed plant cells (Flavell *et al.,* 1994). The selectable marker gene encodes an enzyme which inactivates a herbicide, an antibiotic, metabolic inhibitor or a plant hormone. Only the plant cells which have received the marker gene will survive when placed in a selection medium containing the herbicide or antibiotic. These antibiotic and herbicide resistance marker genes are often used for stable transformation (Bowen, 1993). When these genes are expressed, the transformed cells grow and survive in the selection medium but the untransformed cells eventually die.

The most commonly used selectable marker genes in rice are phosphinotricin acetyl transferase (*bar*) and hygromycin phosphotransferase (*hpt or hph*) genes. The *bar* gene confers resistance to herbicidal compounds, such as, phosphinothricin, glufosinate and bialophos (Dekeyser *et al.*, 1989; Hiei *et al.*, 1997). The *hpt* gene confers resistance to an antibiotic called hygromycin B (Gritz and Davies, 1983). Hygromycin B, isolated from *Streptomyces hygroscopicus*, is a strong inhibitor of polypeptide synthesis of eukaryotic cells (Gonzalez *et al.*, 1978) and has been widely used in selecting transformed protoplast or calli cells in rice transformation. The *hpt* gene has been incorporated in various rice varieties such as Taipei 309 and Nipponbare (Hayashimoto *et al.*, 1990), Labelle (Li *et al.*, 1992b and c), and Khazar and Tarom Molaii (Ghareyazie, 1995; Ghareyazie *et al.*, 1997).

ii. Selectable-marker free plants

With the increasing public concern (though not scientifically supported) regarding the proliferation of antibiotic resistance genes used as selectable markers in the production of transgenic plants, selectable marker-free transgenic rice plants are desirable. Two techniques, namely, the Cre/LoxP system of P1 bacteriophage (Odell *et al.*, 1990) and the FLP/FRT system (Lloyd and Davis, 1994) have been developed to selectively remove the selectable marker gene from the transgenic progeny. The transgenic lines having the selectable marker gene were flanked by recombinase target sequences and then the plants are crossed to screen for excision of the selectable marker gene and the segregation away from the recombinase gene.

A novel selection strategy for the selection of transgenic plants without the use of antibiotics or herbicides has been initially reported in sugar beet (Joersbo *et al.*, 1998) and maize (Negrotto *et al.*, 2000). The positive selection system uses the *pmi* gene which encodes for phosphomannose isomerase that coverts mannose-6-phosphate to fructose-6-phosphate. Only transformed cells were capable of using mannose as carbon source. The mannose selection system was devoid of the

disadvantages of antibiotic or herbicide selection. Successful selection using mannose as a selective agent was reported in transgenic japonica rice (Lucca *et al.*, 2001; He *et al.*, 2004) and indica rice (Datta *et al.*, 2003).

iii. Reporter genes

The reporter gene codes for a visible indicator such as color, luminescence or fluorescence which can be observed from the transformed cell, hence, the gene product 'reports' the presence of the gene. The reporter gene is used for analysing the promoter activities, detecting or quantifying the efficiency of gene transfer and following the inheritance of the gene in the subsequent generations. The reporter gene is always used in gene regulation and expression studies in transient transformation or stable transformation (Birch and Bower, 1994). The popular reporter genes used are the following: green fluorescent (*gfp*) (Chalfie *et al.*, 1994; Sheen *et al.*, 1995; Reichel *et al.*, 1996), luciferase (*luc*) (Ow *et al.*, 1986), chloramphenicol acetyl transferase (*cat*) (Herrera-Estrella *et al.*, 1983) and β-glucuronidase (*gusA*) genes (Jefferson , 1987).

The most commonly used reporter gene in rice is the bacterial *gusA* gene (Jefferson, 1987; Zhang and Wu, 1988; Matsuki *et al.*, 1989; Shimamoto *et al.*, 1989). The *gusA* gene, isolated from *E. coli*, encodes a hydrolase β-glucuronidase which catalyzes the cleavage of a wide range of β-glucuronides (Jefferson, 1987). The β-glucuronidase enzyme can be assayed histochemically or quantitatively (Jefferson, 1987; Jefferson *et al.*, 1989). In histochemical assays, 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-gluc) is added as the substrate and the resultant product becomes visible.

The *gusA* gene is often used to evaluate the transformation conditions as well as the plasmid constructs. Although use of the *gusA* gene has desirable qualities such as high sensitivity, low background endogenous activity and absence of interference with plant metabolism, assaying GUS activity is a destructive process (Upadhyaya *et al.*, 2000).

iv. Agronomically-important genes

Researches are aimed at improving yield and quality while lowering input/production costs through the development of genetically engineered rice (Tai, 2003). Efforts are being made to reduce production costs for the producer and increase the value and/or improve quality for the consumer. Using genetic transformation, agronomically-important genes have been introduced into rice for improvement of nutrition and grain quality, yield enhancement, herbicide resistance, insect resistance, disease resistance and stress tolerance.

Nutrition and grain quality

Rice in its preferred milled form provides relatively little nutritional value because it lacks many vitamins and important micronutrients. In the early 1990s, specific expression of the phytoene synthase (*psy*) from daffodil in the endosperm tissue of rice resulted in the accumulation of phytoene, a precursor to β-carotene not normally found in the rice endosperm (Burkhardt *et al.,* 1997). Transgenic rice plants containing the phytoene desaturase (*crtI*) gene from *Erwinia uredovora* resulted in the production of β-carotene and other carotenoid compounds in rice endosperm (Ye *et al.,* 2000). Datta *et al.,* (2003) introduced a combination of genes, such as phytoene synthase (*psy*) *and* lycopene b cyclase (*lcy*) genes, which enabled the biosynthesis of provitamin A in the endosperm of several indica rice cultivars adapted to diverse ecosystems of different countries. Another area of interest is increasing the iron content of rice grains and improving the uptake of the iron available in rice (Gura, 1999).

Recently, Krishnamurthy and Giroux (2001) introduced the wheat puroindoline genes (*pinA* and *pinB*) into rice to examine their ability to change the grain texture. The expression of *pinA* and/or *pinB* genes reduced grain hardness and produced flour having reduced starch damage and an increase percentage of fine particles.

Yield enhancement

There has been a great interest in engineering metabolic pathways, such as C_4 metabolism, from other plants into rice to increase the physiological efficiency of rice. Transgenic rice plants expressing either the phosphoenolpyruvate carboxylase or the pyruvate, orthophosphate dikinase enzymes from maize show an increased photosynthetic capacity over untransformed control plants (Ku *et al.*, 1999). Initial yield trials of these transgenic plants indicated increased yield due to increased tiller number. This study suggests that introduction of C_4 photosynthetic enzymes may be an effective way to increase rice yields.

Herbicide resistance

Initial studies in rice involved the transfer of selectable marker genes such as *bar* gene which provide protection against herbicidal compounds. Transgenic rice plants have glyphosate resistance via the transfer of the *CP4* gene (Rood, 2000, 2001) and glufosinate resistance via the transfer of the *bar* gene which confers bialophos resistance (Christou *et al.*, 1991; Cao *et al.*, 1992; Datta *et al.*, 1992; Duan *et al.*, 1996; Sankula *et al.*, 1997; Rood, 2000, 2001).

Insect resistance

Since the early 1990s, researchers have been working on the introduction of insect-resistance genes into rice. Transgenic rice plants with the *Bacillus thuringensis* (*Bt*) gene encodes toxins which have specific biological activity against lepidopteran insects, such as the leaffolder (Fujimoto *et al.*, 1993; Wunn *et al.*, 1996), striped stem borer (Fujimoto *et al.*, 1993; Wunn *et al.*, 1996; Cheng *et al.*, 1998) and yellow stem borer (Nayak *et al.*, 1997; Wunn *et al.*, 1996; Cheng *et al.*, 1998). Field tests were recently conducted with an elite Chinese commercial hybrid rice transformed with the *Bt* gene (Tu *et al.*, 2000). Trangenic rice plants showed a high level of protection against both natural and introduced infestations of leaffolder and yellow stem borer without a reduction in yield. Expression of

potato proteinase inhibitor II (*pin II*) gene (Duan *et al.*, 1996) has also enhanced resitance in rice against stem borers.

Disease resistance

Several strategies for engineering resistance to pathogens have been adopted in rice. The use of constitutive expression of rice chitinase gene has led to enhanced resistance of rice against sheath blight (Datta *et al.*, 2001). Expression of *Xa-21* gene in transgenic rice plants has also enhanced resistance against bacterial blight (Datta *et al.*, 2002; Zhao *et al.*, 2000). Expression of the coat protein of rice stripe virus (Hayakawa *et al.*, 1992), the RNA-dependent RNA polymerase of rice yellow mottle virus (Pinto *et al.*, 1999) and a ribozyme targeted against the rice dwarf virus (Han *et al.*, 2000) are various strategies to obtain resistance against viral pathogens of rice. Expression of genes from other organisms have increased resistance of rice to *Xanthomonas oryzae,* the causal agent of bacterial leaf blight disease (Sharma *et al.*, 2000; Tang *et al.*, 2001).

Stress tolerance

Genetic engineering efforts to enhance the tolerance of rice to abiotic stress due to salt, drought and cold have been recently reported. Transgenic rice plants expressing genes for the biosynthesis of glycine betaine, an osmoprotectant. Transgenic rice plants expressing the choline oxidase (*codA*) gene recovered from salt stress more rapidly than wild type plants and the expression of the choline oxidase provided more tolerance to inhibition under salt and cold stress conditions. Rice transformed with the betaine aldehyde dehydrogenase gene from barley showed enhanced resistance to salt and temperature stress (Kishitani *et al.*, 2000). Rice plants expressing the arginine carboxylase gene from oat shows salinity stress tolerance (Roy and Wu, 2002). Overexpression of genes encoding glutamine synthetase (Hoshida *et al.*, 2000) and calcium-dependent protein kinase (Saijo *et al.*, 2000) enhanced stress tolerance in rice. Transgenic rice plants expressing the gene encoding nicotianamide aminotransferases exhibited

improved tolerance to low-iron conditions and yielded about four times more than the untransformed control plants.

1.2.3.5 Techniques in molecular analysis of the transgenic plants

Molecular analysis of transgenic plants requires the use of several techniques such as polymerase chain reaction (PCR), Southern blot, reverse-transcriptase-PCR (RT-PCR), northern blot, enzyme assay and western blot (Gardner *et al.*, 1991). PCR and Southern blot are used to analyze the genomic DNA. RT-PCR and northern blot are used to analyze the transcript and mRNA. Enzyme assay and western blot are used to analyze the enzyme or protein. For example, western blot analysis showed that the BT protein was present in the leaves of the transgenic *Bt* rice plants which indicate the expression of the *Bt* gene. The transgenic *Bt* rice plants showed 100% mortality against the yellow stem borer which indicate that the BT protein is functional (Ghareyazie *et al.*, 1997). PCR and Southern blot determine the inheritance, segregation, co-segregation or deletion of the gene. The latter also determines the number of copies introduced in the genome, number of independent events and number of integration sites. RT-PCR, northern blot, enzyme assay and western blot determines the expression of the gene.

a. Polymerase chain reaction

Polymerase chain reaction (PCR), invented by K.B. Mullis in 1983, is a technique for the synthesis of large quantities of specific DNA segments (Stenesh, 1989). PCR involves a series of repetitive cycles of DNA denaturation, annealing of primers to single stranded DNA and primer extension catalyzed by *Taq* DNA polymerase to amplify a specific DNA segment (Gardner *et al.*, 1991). The DNA is denatured in high temperature such as 94°C. During annealing, the primers bind to the site in the DNA which is complementary to their sequence. During elongation or extension, the *Taq* polymerase binds to the primer-DNA complex and adds corresponding dNTPs to the 3' OH end of the primer for replication. The repeated temperature cycling is easily and reproducibly performed by a

programmable thermal cycler. The PCR products can be visualized by running them on an ethidium bromide-stained agarose gel and viewing the gel on a uv transilluminator.

The use of PCR in the evaluation of transgenic plants and progeny, for the presence of foreign genes, has many advantages. PCR is a fast, reliable method, requiring a little amount, in nanograms or picograms, of rice genomic DNA and not requiring the use of radioactive material. PCR is easy to do, requiring little time to see results, allowing the screening of plants as early as seedling stage, hence, PCR is very useful in analyzing the presence of the gene in a large number of putative transgenic plants with relative ease. However, the integration pattern of the genes introduced in rice are not shown so the independent transformants cannot be identified using PCR analysis.

b. Southern blot

Southern blot or DNA gel blot, invented by E. Southern in 1975, is a molecular technique involving the capillary transfer of DNA fragments, that are separated by agarose gel electrophoresis and denatured *in situ*, to a solid support such as a nitrocellulose filter or nylon membrane (Stenesh, 1989).

The use of Southern blot has the following disadvantages and advantages (Ghareyazie, 1995). It is laborious and very demanding of labor and materials, requires a high quantity (5.5 μg) and high quality of DNA, takes several days to get a result and involves the use of a radioactive isotope or non-radioactive isotope. Nevertheless, this technique has been widely used in the evaluation of transgenic plants by many workers because of its advantages. It can detect the presence of foreign genes, the number of copies of the foreign gene and the number of independent events occurring in transformation in the putative transformed plants and its progenies.

c. RT-PCR

RT-PCR involves reverse transcribing the RNA into cDNA using a reverse transcriptase prior to PCR amplification (Finch, 1994; Turner *et al.*, 2000). RT-PCR is being used increasingly in plants to study the aspects of gene expression (Finch, 1994). The expression of the gene in different parts and stages of the transgenic plants can be examined using RT-PCR analysis.

1.3 Targets of the research

1.3.1. Address the problem of poor grain filling of New Plant Type rice

Breeding of the NPT rice with large panicles and few tillers aimed to break the yield barriers of 10t/ha in the tropics. However, field test at several sites between 1994 and 1997 indicated low yields from NPT lines due to low biomass production, pest susceptibility and poor grain filling (Peng *et al.*, 1998). Although NPT rice was designed to have large panicle which has 200-250 grains per panicle, all these grains are not 100% filled and many NPT lines have poor grain filling (Peng *et al.*, 1994; Virk and Khush, 2002). Hence, the grain yield is lower in many NPT lines due to poor grain filing (Peng *et al.*, 1999). Poor grain filling might be due to compact arrangement of spikelets on the panicle (Khush and Peng, 1996), a limited number of large vascular bundles for assimilate transport and source limitation due to early leaf senescence (Ladha *et al.*, 1998).

1.3.2. Leaf senescence in rice plants

Leaf senescence is an actively ordered process that involves the synthesis of new RNAs and proteins and results in highly coordinated changes in metabolism (Nooden and Leopold, 1978, Buchanan-Wollaston, 1997, Gan and Amasino, 1997 and Nam, 1997). The nutrients are remobilized to actively growing regions such as new leaves, developing seeds and fruits (Biswal and Biswal, 1999). The leaf cells undergo an orderly, sequential disassembly or breakdown of macromolecules

such as proteins, nucleic acids and lipids (Biswal and Biswal, 1999). As a result of the prominent chloroplast breakdown, the leaves lose chlorophyll and consequently visibly marked by yellowing, hence, leaf senescence is generally measured in terms of chlorophyll loss (Nooden and Leopold, 1978).

Leaf senescence is induced by internal factors such as seed development (Nooden, 1988) and by external factors such as insufficient nutrients, pathogen infection and high temperature. Monocarpic senescence occurs in rice plants wherein senescence and death of the plants happen after the reproductive stage, flowering and production of fruits/grains (Nooden and Leopold, 1978).

1.3.3 **Approaches to delaying leaf senescence to enhance rice grain filling**

Leaf senescence is the last phase of leaf development wherein the nutrients are recycled to the new leaves and developing seeds (Lohman *et al.*, 1994; Biswal and Biswal, 1999). Since the top three leaves are the main source of photosynthates during rice grain filling (Yoshida, 1971), increasing the photosynthetic life span of these leaves by delaying leaf senescence may enhance grain filling. Simulation modelling suggests that prolonging grain-filling duration will result in an increase in grain yield. Leaf senescence can be delayed by exogenous application of cytokinins (Ray and Choudhuri, 1981) and by genetic manipulation of cytokinins (Cao, 2001; Lin *et al.*, 2002).

Leaf senescence is affected by the level of endogenous cytokinins. Cytokinins are plant hormones generally considered to induce cell division and affect some other physiological processes including retardation of leaf senescence (Davies, 1995). Cytokinins accumulating in developing seeds shortly after anthesis (Saha *et al.*, 1986; Morris *et al.*, 1993; Yang et al. 2003) affect the number of endosperm cells at early grain filling stages (Jones *et al.*, 1992, Yang J.C. et al. 2003). Cytokinins delay leaf senescence (Richmond and Lang, 1957, Nooden *et al.*, 1997) by promoting chloroplast development (Caers and Vebdrig, 1986), prolonging activity of photosynthetic apparatus (Shu-Quing et al., 2004)

and inhibiting dismantling of chloroplasts and their components (Synkova et al. 1997). The biosynthetic and metabolic pathway for cytokinins is shown in Figure 1.2 The concentration of endogenous cytokinins declines in plant tissues as senescence progresses (Van Staden *et al.,* 1988) probably due to decrease in the supply of cytokinins from the roots. This decline can be a major factor in signalling of monocarpic senescence. (Nooden et al. 1990).

a. Conventional approach to delaying leaf senescence

Maintaining the assimilation activity of leaves at a high level for a long period during the ripening stage results in an increase of yield by an increase in grain filling percentage. An application of cytokinin was suggested to maintain the function of leaves at a high level after flowering. Exogenous application of cytokinins to rice plants has increased the grain filling and yield by delaying leaf senescence (Ray and Choudhuri, 1981). Application of cytokinin at flowering and after flowering improved grain filling and yield of rice plants which is probably due to increased sink strength or delayed leaf senescence. Although the exogenous cytokinin application to the whole plant permits delivering the cytokinins at the specific stage of plant development, it does not ensure targeting (Kaminek *et al.,* 2003). There is also a risk of side effects in inhibition of root development when cytokinins are splashed down into the rhizosphere. Furthermore, it is a costly operation to have large-scale exogenous application of cytokinins.

b. Transgenic approach to delaying leaf senescence

Genetic manipulation of cytokinin levels by expressing the isopentenyl transferase (*ipt*) gene which encodes an enzyme involved in cytokinin biosynthesis is a more potentially efficient method of delaying leaf senescence (Kaminek *et al.,* 2003). The *ipt* gene, from *Agrobacterium tumefaciens*, encodes an isopentenyl transferase enzyme which catalyzes the first and rate-limiting step in cytokinin biosynthesis (Akiyoshi *et al.,* 1984). Table 1.6 shows that IPT system (using *ipt* gene under the control of different types of promoters) and non-IPT systems have

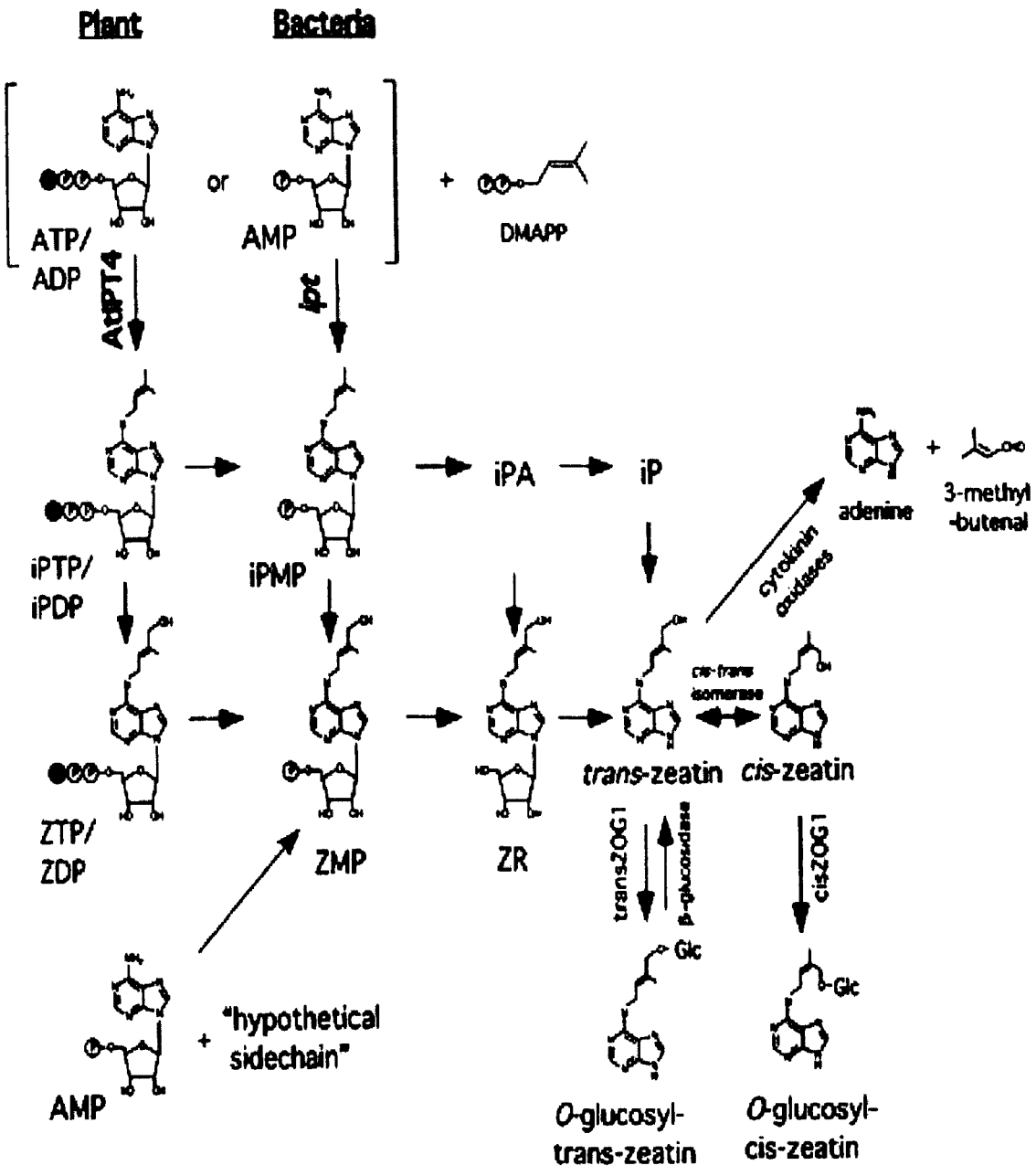

Figure 1.2. Proposed biosynthetic and metabolic pathway for cytokinins. Left, the proposed biosynthesis of zeatin tri-/diphosphate in *Arabidopsis*. Both ADP and ATP are likely substrates for the plant IPT enzyme, and these and their di- and triphosphate derivatives are indicted together (e.g. ATP/ADP). The biosynthesis of cytokinins in bacteria (e.g. *A. tumefaciens*) is compared next to it. Right, Several possible modifications and the degradation of zeatin (adapted from Haberer and Kieber, 2002).

Table 1.6. Transgenic plant systems that have resulted in altered cytokinin levels (Adapted from Gan and Amasino, 1996)

Systems	Source of promoter	Transgenic *species*	References
A. IPT systems			
1. IPT native promoter	From pTi15955	*Nicotiana tabacum*	Schumulling *et al.*, 1989
	From pTiC58	*Solanum tuberosum*	Ooms *et al.*, 1991
	From pTi15955	*N. tabacum*	Yushibov *et al.*, 1991;
	CaMV53S	*N. tabacum*	Beinsberger *et al.*, 1992
2. Constitutive expression		*Lycopersicon esculentum*	Zhang *et al.*, 1995
	CaMV35 S promoter disrupted by Ac transposon	*N. tabacum*	Groot *et al.*, 1995
3. Transposition		*N. plumbaginifolia*	Smigocki and Owens, 1988
	Unknown	*Cucumis sativa*	
4.Random insertion		*N. tabacum*	
5. Inducible	Maize hsp70	*N. tabacum*	Estruch *et al.*, 1991, 1993
	Drosophila hsp 70	*N. tabacum*	Hewelt *et al.*, 1994
a. Heat	Soybean HS6871	*Arabidopsis*	

	Soybean Gmhsp17.5-E	*thaliana* *N. tabacum*	Medford *et al.*, 1989
b. Wounding	Potato proteinaise inhibitor II-K gene	*N. plumbaginifolia*	
c. Light	Pea rubisco small subunit gene	*N. tabacum*	Schumulling *et al.*, 1989
d. Tetracycline		*N. tabacum*	
e. Copper	Tetracycline-dependent CaMV35S	*N. plumbaginifolia*	Smigocki, 1991
		N. tabacum	Smart *et al.*, 1991
	Yeast copper-metallothionein regulatory system	*N. tabacum*	Ainley *et al.*, 1993
6.Tissue-specific		*N. tabacum*	
a. Elongating region		*N. tabacum*	Smigocki *et al.*, 1993
b. Fruit-specific			
c. Ovary-preferential	Soybean SAUR gene	*N. tabacum*	Beinsberger *et al.*, 1992
		L. esculentum	Faiss *et al.*, 1997
7.Development-specific	Tomato 2A11 gene	*L. esculentum*	
	Tomato unknown gene		McKenzie *et al.*, 1998
Senescence-specific		*N. tabacum*	
B. Non-IPT systems	*Arabidopsis* SAG12		
		N. tabacum	Li *et al.*, 1992a
1. Homeobox protein		*N. tabacum*	Martineau *et al.*, 1994
a. NTH15	CaMV 35S	*N. tabacum*	
b. OSH1	CaMV 35S	*N. taabcum*	Martineau *et al.*, 1994
	NOS	*L. esculentum*	
	Arabidopsis SAG12		

c. Knotted 1 2. Small GTP binding protein	CaMV 35S		Gan and Amasino, 1995 Tamaoki *et al.*, 1997 Kusaba *et al.*, 1998 Ori *et al.*, 1999 Sano *et al.*, 1994

produced transgenic plants which have resulted in altered cytokinin levels (Gan and Amasino, 1996). Expression of the *ipt* gene fused to heat-shock or tissue-specific promoters produced increased levels of cytokinins in plants or delayed leaf senescence (Medford *et al.*, 1989; Schumulling *et al.*, 1989; Smart *et al.*, 1991; Smigocki, 1991; Beinsberger *et al.*, 1992; Li *et al.*, 1992a; Ainley *et al.*, 1993; Smigocki *et al.*, 1988; Van Loven *et al.*, 1993; Martineau *et al.*, 1994; Thomas *et al.*, 1995). However, the transgenic plants are morphologically and developmentally abnormal (Klee, 1994) which gives little potential for agricultural application. Gan and Amasino (1995) have developed an autoregulatory senescence-inhibition system in which a highly senescence-specific *SAG12* promoter was fused to the *ipt* gene. The *SAG12* promoter, from *Arabidopsis thaliana,* directs the expression of the *ipt* gene at the start of leaf senescence and subsequently elevates the cytokinin content so that it prevents the leaf from senescing, which in turn leads to down-regulation of the senescence-specific promoter, thereby, preventing the accumulation of a very high level of cytokinins. The transgenic tobacco plants expressing the P_{SAG12}-*IPT* gene showed a markedly delayed leaf and floral senescence, increased biomass and seed yield without any abnormality in plant growth and development. Hence, this autoregulatory senescence-inhibition system has a great potential in agricultural and horticultural applications.

The *ipt* gene fused to the senescence-specific *SAG12* promoter, expressed at the beginning of leaf senescence (Gan and Amasino, 1995), is introduced into rice and explored for site-specific and developmentally-specific accumulation of cytokinins. Since the exogenous application of cytokinins is too costly and there is tight regulation regarding chemical application then genetic manipulation of cytokinin levels using the *ipt* gene to delay leaf senescence and possibly enhance grain filling was the approach used in this project.

1.3.4 Project's main aims and specific objectives

a. Project's main aims

The project aims to:

1.) determine the rate of leaf senescence and cytokinin levels in the leaves of NPT rice plants grown in the field

2.) transform rice with the *isopentenyl transferase (ipt)* gene under the control of a senescence-specific SAG12 promoter

3.) isolate the rice homologue of the SAG12 promoter

b. Project's specific objectives

This research project addressed the following objectives:

1. To determine the changes in chlorophyll content in the top three leaves of field-grown NPT lines during the grain filling period.
2. To determine the rate of senescence in the top three leaves of NPT lines and its relationship with grain filling percentage and grain yield.
3. To determine the changes in cytokinin levels in the top three leaves of NPT lines and its relationship with the chlorophyll content.
4. To transform NPT rice with SAG12-*gus* and SAG12-*ipt* gene using biolistic transformation.
5. To determine the integration, expression and inheritance of the *gus* and *ipt* genes in the transgenic SAG12-*gus* and SAG12-*ipt* plants
6. To determine the grain filling percentage and the levels of cytokinins in the leaves of the transgenic SAG12-*ipt* plants.
7. To isolate the homologue of the *SAG12* promoter in NPT rice.

Chapter 2. Changes in chlorophyll contents, rate of senescence and cytokinin levels in the top three leaves of New Plant Type rice during grain filling

2.1 Introduction

Leaf senescence affects the supply of photo-assimilates to the developing seeds (Nooden, 1988). The top three leaves of rice are the main sources of photo-synthates producing about 80% of the net whole plant photosynthesis during the grain filling period (Yoshida and Cock, 1971). Hence, a rapid senescence of these leaves may significantly affect grain filling. Rapid and slow senescing lines differing in rate of chlorophyll decline were identified in maize, sorghum and rice using a SPAD meter (Park *et al.*, 1993; Thomas and Howarth, 2000). The grain yield was higher in the slow-senescing lines than in the fast-senescing lines in sorghum (Ambler *et al.*, 1992; Thomas and Howarth, 2000) and in rice (Soejima *et al.*, 1995) suggesting that the delay in leaf senescence may increase grain yield due to prolongation of period of photo-assimilate production and their transport to the grains.

Leaf senescence is affected by the level of endogenous cytokinins (CKs) supplied by the roots, through the xylem sap. Measurements show that the concentrations of endogenous cytokinins decline in plant tissues as senescence progresses (Van Staden *et al.*, 1988). Soejima *et al.*, (1995) reported that the CK level in the xylem sap was higher in the slow-senescing and high-yielding Akenohoshi rice cultivar than the standard Japanese Nipponbare cultivar. Furthermore, the flux of total cytokinins per plant or per unit leaf area was significantly correlated with the chlorophyll content in the rapidly senescing third leaves but not in flag leaves and second leaves. The CKs coming up through the xylem from the roots play an important role in maintaining the foliage and a decline in the supply of CKs from the roots can be a major factor in monocarpic senescence (Garrison *et al.*, 1984).

CKs delay leaf senescence (Richmond and Lang, 1957) which affect grain filling and yield. Exogenous application of CKs in rice plants increased grain filling and yield (Ray and Choudhuri, 1981) and genetic manipulation of CKs in rice plants delayed leaf senescence and increased the yield (Cao, 2001). However, enhanced leaf senescence has also increased grain filling and yield of rice plants subjected to water deficit during grain ripening (Yang *et al.*, 2002b). Rapid senescing rice cultivars have higher grain filling percentage than slow senescing rice cultivars (Park *et al.*, 1993).

The New Plant Type (NPT) rice was designed to have larger panicle size (up to twice as many spikelets per panicle as found in the semi-dwarf indica rice variety such as IR72). In the dry season of 1996, the characteristics of panicles and flag leaves of IR72 and NPT line IR65598-112-2 were compared (Ladha *et al.*, 1998). IR72 formed a large number of panicles whereas IR65598-112-2 had fewer panicles but much larger size of panicles. The area of the flag leaf and Rubisco content of the NPT line were twice higher than in IR72. However, IR72 had 12% unfilled grains and a yield of 9.7 t/ha whereas IR65598-112-2 had 34% unfilled grains and gave 9.2 t/ha yield. Reducing the percentage of unfilled grains in the NPT line to the level of 12%, as in the case of IR72, might lead to a higher yield of NPT rice. It was not clear what causes the high percentage of unfilled grains in IR65598-112-2. The possible causes of poor grain filling are the following: the late grains may fail to fill due to the competition between early grains and late grains for nutrients, compact arrangement of spikelets on the panicle (Khush and Peng, 1996), a decline in photosynthetic capacity, an inadequate rate of transport of photosynthate from leaf to grain, limited viability of late-filling spikelets or early leaf senescence (Ladha *et al.*, 1998).

Several lines of research have been carried out to investigate these failings. One possibility is that poor grain filling might be due to the source limitation brought about by a problem relating to leaf senescence (Ladha *et al.*, 1998). It is not known whether NPT rice displays a sudden degradation of the photosynthetic apparatus or whether senescence occurs over a prolonged period.

In general, there are two possible schemes for efficient supply of developing seeds with photoassimilates, namely, (1) delay of leaf senescence and extension of production and translocation of photoassimilates to the seeds and (2) enhanced leaf senescence caused by the sudden degradation of photosynthetic apparatus and re-utilization of metabolites by developing seeds. In the first scheme, the delay of leaf senescence increases grain yield. Rikuu 132 rice cultivar and Akenohoshi rice cultivar (Soejima *et al.,* 1995), and in sorghum (Ambler *et al.,* 1992; Thomas and Howarth, 2000) are slow-senescing and have high yield which suggests that the delay in leaf senescence may increase grain yield due to prolongation of period of photoassimilate production and their transport to the grains. In the second scheme, rapid senescence increases grain yield. Delayed senescence, which is induced by either too much nitrogen fertilizer or an adoption of strong lodging-resistant varieties that stay "green" for too long, delays such remobilization and can lead to slow grain filling (Yang *et al.,* 2002b). However, reports showed that early senescence induced by a moderate water deficit during grain-filling period has enhanced the remobilization of stored assimilates and accelerate grain-filling of wheat (Yang *et al.,* 2001a) and rice (Yang *et al.,* 2001b).

The onset of leaf senescence is induced by a variety of external factors, such as shading, extreme temperature, nutrient deficiency, drought and pathogen infection and by developmental processes such as seed development, age and phytohormones. Among the phytohormones, abscisic acid promote while cytokinins (CKs) inhibit rice leaf senescence. Leaf senescence is affected by the level of CKs supplied by the roots, through the xylem sap. Leaf senescence is correlated with a decline in endogenous cytokinin levels in the leaves (Van Staden *et al.,* 1988). Milyang 23 rice cultivar showed a higher level of endogenous CKs and a slower leaf aging process whereas Nanging 11 rice cultivar showed a higher level of endogenous abscisic acid and a more accelerated aging process. Soejima *et al.,* (1995) reported that the level of CKs in the xylem sap was higher in the slow-senescing and high-yielding Akenohoshi rice cultivar than the standard Japanese Nipponbare cultivar. Furthermore, the flux of total cytokinins per plant

or per unit leaf area was significantly correlated with the chlorophyll content in the rapidly senescing third leaves but not in flag leaves and second leaves. The CKs coming up through the xylem from the roots play an important role in maintaining the foliage and a decline in the supply of CKs from the roots can be a major factor in monocarpic senescence (Garrison *et al.*, 1984).

Soejima *et al.*, (1995) studied the changes in chlorophyll contents of leaves and levels of cytokinins in root exudates during ripening of Akenohoshi and Nipponbare rice culrivars. They found that the chlorophyll contents of the flagleaf, the second and third leaves decreased in a sequential manner in both cultivars during the grain filling period. The chlorophyll content of the third leaves of Akenohoshi was significantly correlated with the flux of total CKs per plant or per unit area which suggests that the larger amounts of CKs caused the slow senescence of leaves of Akenohoshi during the grain filling period.

The changes in chlorophyll content, rates of senescence and CK levels in the leaves of NPT rice lines during the grain filling period have not been studied so far. The relationship of the rate of senescence to the grain filling percentage and grain yield in NPT lines were not yet known. The purposes of this study were (1) to determine the scheme used by NPT rice for efficient supply of photoassimilates to the developing seeds, (2) to compare the changes in chlorophyll contents and CK levels in the top three leaves of slow-senescing and fast-senescing NPT lines during grain filling, (3) to determine the correlation between the rate of leaf senescence and grain filling percentage and grain yield and (4) to determine the relationship between the chlorophyll contents and CK levels in the leaves of slow-senescing and fast-senescing NPT lines. The comparison of rates of senescence with changes in CKs levels could provide information about potential involvement of CKs in regulation of leaf senescence in NPT lines.

2.2 Materials and Methods

2.2.1 Plant materials

Experiments were conducted at the International Rice Research Institute (IRRI), Philippines in the wet season of 1999 (June to November). The experimental site was located at 14° 11' N, 12° 15' E, altitude 21 m. Four rice NPT lines (IR68552-100-1-2-2, IR69092-57-3, IR67962-40-6-3-3 and IR67966-44-2-3-2) were grown in the field with a plot size of 5 x 20 m. Twenty-one day old seedlings were transplanted in June at a hill spacing of 0.15 m x 0.10 m with one seedling per hill. A Maahas clay soil (Andaqueptic Haplaquoll) was used. Nitrogen (40 kg ha^{-1} as urea), P (20 kg ha^{-1} as single superphosphate) and K (40 kg ha^{-1} as KCl) were incorporated in the soil before transplanting. Nitrogen was applied at midtillering (40 kg ha^{-1}) and at panicle initiation (20 kg ha^{-1}).

2.2.2 Measurement of the chlorophyll content

The chlorophyll content was measured in the middle portion of the leaf blade of the top three leaves of four NPT lines using a hand-held SPAD chlorophyll meter (Minolta SPAD-502). The SPAD values were measured in the top three leaves of thirty plants from each of the four NPT lines at 0, 1, 2 and 3 weeks after flowering (WAF). The rate of leaf senescence was determined by taking the difference in SPAD values between 0 and 3 WAF.

2.2.3 Evaluation of yield components and grain yield

The yield components such as number of filled grains per m^2, number of half-filled grains per m^2, empty grains per m^2, total number of grains per m^2, percentage of filled grains per m^2, spikelets per panicle, and 1000-seed weight were taken from 8 panicles of plants. The percentage of filled grains was calculated as the number of filled grains/total number of grains x 100. Grain yield, taken from plants in 5 m^2 area, was computed using the following formula:

Grain yield (t/ha) = (yield per area [in grams]/5 m^2 area) * (fresh weight of seeds [in grams]/weight of these seeds at 14% moisture content [in grams]) *10000/1000000.

2.2.4 Cytokinin extraction, purification and HPLC/MS analysis

The top three leaves (flag leaf, second leaf and third leaf) of three plants each of a fast-senescing NPT line (IR68552-100-1-2-2) and a slow-senescing NPT line (IR67966-44-2-3-2) were sampled at 0, 1, 2 and 3 WAF for CK analysis. CKs in these leaves were extracted and purified using the method of Dobrev and Kaminek (2002). Leaf samples (about 1 gram fresh weight [FW]) were powdered in a mortar in liquid nitrogen and extracted overnight in 5 ml of cold extraction solution (-20°C) of methanol/water/formic acid (15/4/1, v/v/v, pH 2.5) containing deuterium labelled CK internal standards (Apex, Honiton, Devon, UK). Each of the deuterium (^2H)-labelled standards, namely, [$^2H_5$] Z, [$^2H_5$] DHZ, [$^2H_6$] iP, [$^2H_5$] ZR, [$^2H_5$] DHZ9R, [$^2H_6$] iP9R, [$^2H_5$] Z7G, [$^2H_5$] Z9G, [$^2H_5$] ZOG, [$^2H_5$] ZROG, [$^2H_6$] iP7G, [$^2H_6$] iP9G, [$^2H_5$] ZRMP, [$^2H_5$] DHZRMP and [$^2H_6$] iP9RMP was added at 50 pmol per sample. After separation of solids by centrifugation (20,000 x *g*, 15 minutes) and re-extraction of the sediment with the same solvent (5 ml), CK bases, ribosides and glucosides were separated from IAA, ABA and CK ribotides on a mixed-mode (reverse-phase and cation-exchange) MCX column (Waters, Milford, MA, USA) by sequential elution using solvents of increasing methanol and ammonium concentration. The CK bases and ribosides were eluted with 0.35 M ammonium in 60% methanol (v/v). The CK nucleotides were eluted with 0.35 M ammonium in water. The eluate from each purification step was evaporated at 40°C under vacuum to dryness. The samples containing the CK nucleotides were dephosphorylated for 1 hour at 37°C with acid phosphatase (0.6 units per sample). The evaporated samples were suspended in 100 µl of 10% acetonitrile (v/v) and subjected to HPLC/MS analysis.

The CKs were quantified using Quaternary HPLC gradient pump Rheos 200 (Flux Instruments) and HTS-PAL autosampler (CTC Analytics) linked to an Ion Trap Mass Spectrometer LCQ (Finnigan MAT) equipped with an electrospray interface. Samples (10 µl) were injected on AQUA C_{18} column (2x250 mm, 5 µm, Phenomenex). Samples were eluted with a multilinear gradient of water (A), acetonitrile (B), water (C) and 0.01%,v/v, acetic acid in water (D) at flow rate of 0.2 ml min^{-1}. Analysis started at 10% B and 5% D and these values were kept for 5 min. By the first, ramp B was increased to 17% and D to 15% in 10 min, then B was increased to 50% in 11 min. At the end of the run, B was increased to 85% and D decreased back to 5% and maintained for 4 min. Detection and quantification were carried out using a Finnigan LCQ operated in the positive ion, full-scan MS/MS mode using a multilevel calibration graph with [^{2}H]-labelled CK as internal standards. Due to the high cost of the analysis, the CK analysis beginning from extraction was repeated only twice, which led to relatively high variation of results. Nevertheless, results of the two analyses showed very similar trends. The results presented here represent the mean values.

Deuterium-labelled CKs (D-CKs) are cytokinins which are chemically identical to normal CKs with the only difference that some hydrogens (usually 5-6) in the molecule are replaced by deuteriums. Because deuteriums are twice as heavier than hydrogens, the molecular weight of D-CKs is with 5-6 daltons higher than in the respective normal CK. For example, zeatin has a molecular weight of 219 daltons, whereas D_6-zeatin (with 6 deuteriums) has a molecular weight of 225 (219 + 6) daltons. Chemically and physically, CKs and D-CKs behave almost in the same way. D-CKs are the best internal standards (IS) for CKs because by definition the ideal IS should be chemically and physically very similar to the measured analyte. On the other hand, at the quantification step the IS should be distinguished from the analyte and both should be quantified independently. In this case, D-CKs can be easily distinguished from the normal CKs because they are quantified by mass spectrometry which can easily measure analytes differing in mass. The quantification with IS is made using the following steps:

The IS with known amount (say A) is added to the sample. Upon quantification, the responses of IS and the analyte (X) are measured and their ratio is calculated i.e. response ratio (RR) = response of IS / response of X.

A mixture of known amounts (KnAm) of IS and analyte X is injected separately and their responses were obtained. The constant (K) is calculated using the following formula:

K = (KnAm IS / KnAm X) / (response of IS / response of X).

Finally, calculate the amount of the analyte in the sample (Am X):
Am X = RR * K * A

2.2.5 Statistical analysis

Correlation analysis was used to determine the correlation coefficient between the rate of senescence and grain filling percentage and yield. Correlation analysis was also used to determine the correlation between the chlorophyll content and the level of CKs in the top three leaves of slow- and fast-senescing NPT lines. The significance of differences in the levels of CKs during the aging of the same leaves from 0 to 3 WAF was determined using a single factor analysis of variance (ANOVA).

2.3 Results

2.3.1 Changes in chlorophyll contents in the top three leaves

The changes in chlorophyll content, characterized by SPAD values, in the top three leaves of four NPT lines (IR68552-100-1-2-2; IR69092-57-3; IR67962-40-6-3-3; IR76966-44-2-3-3) during grain filling are shown in Figure 2.1. The chlorophyll content in the three leaves of all these NPT lines gradually decreased with the age of the plants, from flowering to maturity. This decline was slow

between 0 and 1 WAF but more rapid between 1 and 2 WAF. The chlorophyll content decreased at a highest rate in the third leaf followed by the second leaf and then the flag leaf.

2.3.2 Yield components and grain yield

Yield components and grain yield differ among the four NPT lines (Table 2.1). The highest grain yield was observed in IR68552-100-1-2-2 followed by IR67962-40-6-3-3, IR69092-57-3 and IR76966-44-2-3-3. The NPT lines with higher total number of grains have lower percentage of filled grains. The NPT lines with high number of grains is due to the high total number of grains per panicle. The high number of half-filled and empty grains results to high higher percentage of grain filling.

2.3.3 Relationship between the rate of senescence, grain filling percentage and yield

The rate of leaf senescence, grain filling percentage and grain yield of the four NPT lines are shown (Table 2.2). The rate of senescence in the three leaves was highest in IR68552-100-1-2-2, followed by IR69092-57-3, IR67962-40-6-3-3 and IR76966-44-2-3-3. The grain filling percentage was positively and highly significantly correlated with the rate of leaf senescence in the flag leaf, second leaf and third leaf of the four NPT lines (Table 2.3). The grain yield was significantly and positively correlated with the rate of senescence in the flag leaf but not in the second leaf and third leaf.

2.3.4 Changes in the cytokinin levels in the top three leaves

The changes in the levels of CKs in the top three leaves of a fast-senescing NPT line (IR68552-100-1-2-2) and a slow-senescing NPT line (IR67966-44-2-3-2)

Table 2.1. Yield components of the four New Plant Type lines during wet season 1999.

NPT line	Yield (t/ha)	Filled grains/ spikelets per m^2 (no.)	Half-filled grains/ spikelets per m^2 (no.)	Empty grains/ spikelets per m^2 (no.)	Total grains/ spikelets per m^2 (no.)	Percentage of filled grains/ spikelets (%)	Panicles per m^2	Total grains/ spikelets per panicle	1000-seed wt.
IR67962-40-6-3-3	4.97	12938	8930	5044	26912	48.1	213.2	126.2	23.7
IR67966-44-2-3-2	2.94	9967	7398	7404	24769	40.2	231.4	107.0	25.1
IR68552-100-1-2-2	5.47	16300	2864	4097	23261	70.1	239.0	97.3	26.7
IR69092-57-3	4.06	12915	3742	6468	23125	55.8	229.4	100.8	27.1

Table 2.2 The rate of leaf senescence (as measured by the differences in SPAD values determined at 0 and 3 weeks after flowering), grain filling percentage and grain yield of four NPT lines during grain filling.

NPT Line	Rate of leaf senescence in			Grain filling percentage (%)	Grain yield (t ha^{-1})
	Flag leaf	Second leaf	Third leaf		
IR68552-100-1-2-2	9.00	29.00	32.00	70.20	5.47
IR69092-57-3	6.00	19.00	25.00	55.70	4.06
IR67962-40-6-3-3	6.00	10.00	20.00	48.00	4.97
IR67966-44-2-3-2	4.00	6.00	13.00	40.00	2.94

Table 2.3 Correlation coefficient between the rate of leaf senescence, grain filling percentage and grain yield of four NPT lines.

Rate of leaf senescence (as measured by the difference in SPAD values between 0 and 3 weeks after flowering in the)	Grain filling percentage (%)	Grain Yield (t ha^{-1})
Flag leaf	0.97 **	0.90 *
Second leaf	0.99 ***	0.71 ns
Third leaf	0.99 ***	0.81 ns

ns = not significant; * = significant at 0.10 level of probability [r 0.10 (2-tailed), 2 df = 0.90]; ** = significant at 0.05 level of probability [r 0.05 (2-tailed), 2 df = 0.95]; *** = significant at 0.01 level of probability [r 0.01 (2-tailed), 2 df = 0.99]

were observed at 0, 1, 2 and 3 WAF. Twenty two different CKs, identified in the three leaves of both NPT lines, were placed into four different groups of CKs, namely:

(1) active CKs (Figure 2.2)

Z, DHZ, ZR, DHZR, iP, iPR

(2) storage CKs (Figure 2.3)

ZOG, ZROG, DHZROG, DHZOG

(3) inactive CKs (Figure 2.4)

Z7G, DHZ7G, Z9G, DHZ9G, iP7G, iP9G

(4) *cis*-zeatin and its derivative CKs (Figure 2.5)

c-Z7G, c-ZG, c-ZOG, c-ZROG, c-Z, c-Z9R

The content of active CKs at the time of flowering was two-fold higher in the fast-senescing NPT line as compared to slow senescing NPT line but it sharply declined with the leaf age in the flag leaf (Figure 2.2). There was no significant difference in the content of active CKs found in the second and third leaves at flowering between the two NPT lines. A progressive decrease in active CKs was associated with the progressing age of the flag leaf and the second leaf of the fast-senescing NPT line while opposite trend was found in slow-senescing NPT line.

Interestingly, the level of active CKs was increasing with age of the third leaves of both NPT lines and this increase was more pronounced in the fast-senescing NPT line. The level of storage CKs remained at a steady level in the three leaves of the slow-senescing NPT line (Figure 2.3). However, it was increased in the flag leaf, increased and then sharply decreased in the second and third leaves of the fast-senescing NPT line. The two NPT lines differed in the profiles of inactive CKs. The content of inactive CKs remained steady in the flag leaf, increased then sharply decreased in the second and third leaves of the fast-senescing NPT line (Figure 2.4). However, the level of inactive CKs increased in all the three leaves of the slow-senescing NPT line. The *cis*-zeatin and its derivatives CKs remained at a steady level in all the leaves of the slow-senescing NPT line. However, their content was gradually increased in the flag leaf and second leaf but decreased in the third leaves of the fast-senescing NPT line (Figure 2.5).

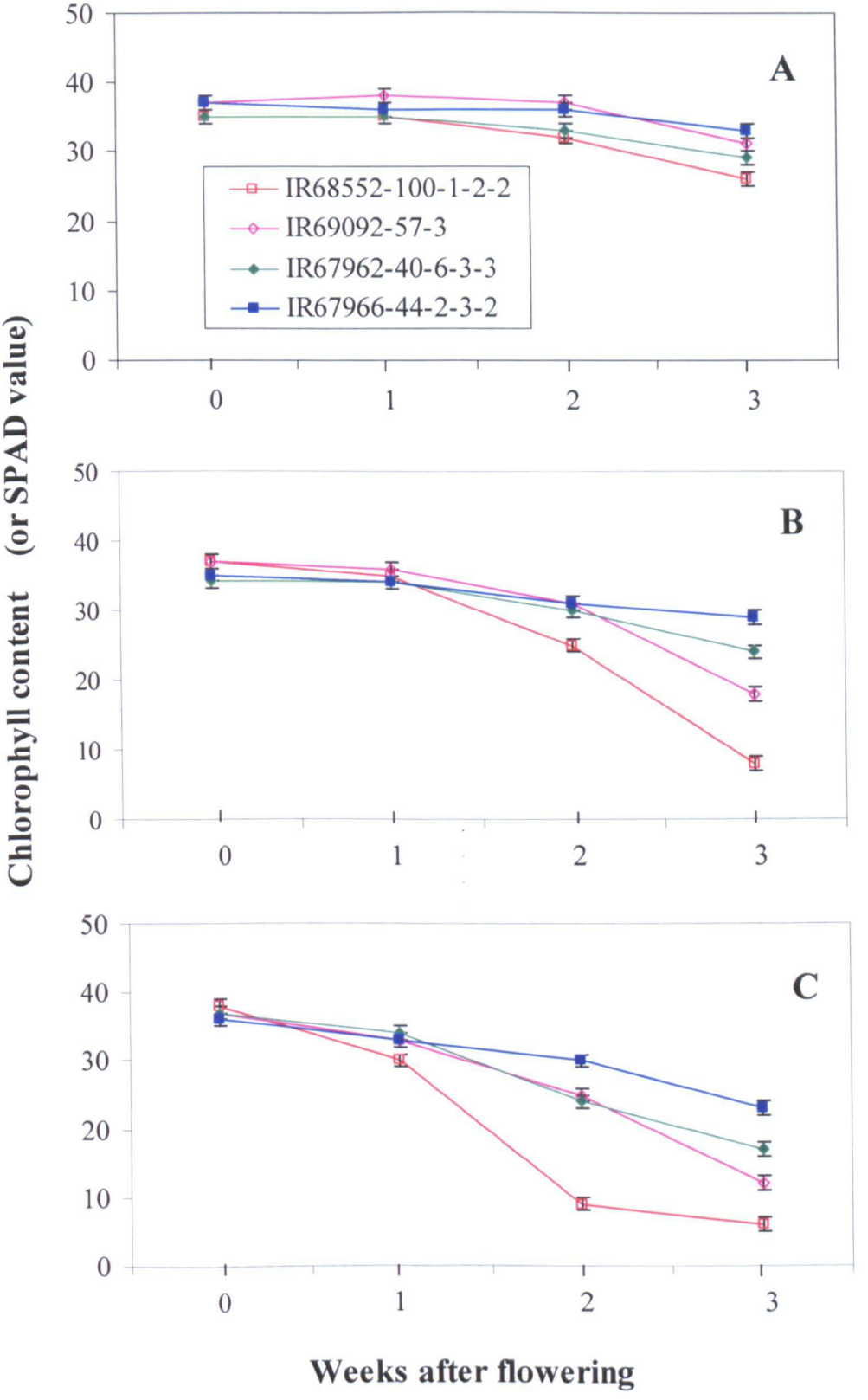

Figure 2.1. Changes in the chlorophyll content (or SPAD value) in the top three leaves, flag leaf (A), second leaf (B) and third leaf (C), of four field-grown NPT lines (IR68552-100-1-2-2, IR69092-57-3, IR67962-40-6-3-3 and IR67966-44-2-3-2) at flowering to maturity. SPAD values are means of 30 measurements from each NPT line. Bars indicate standard errors of the means of 30 measurements.

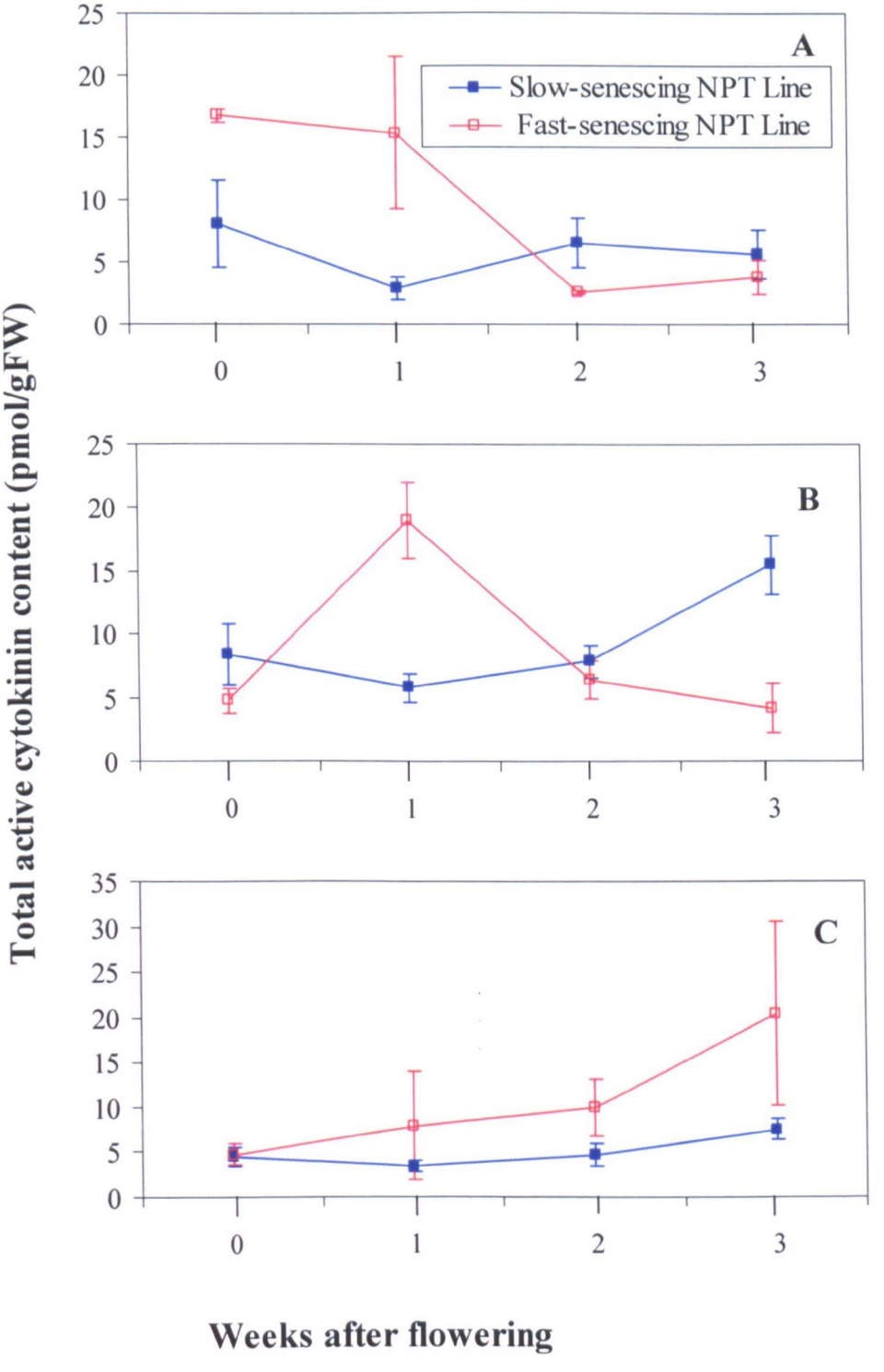

Figure 2.2. The total content of all active cytokinins in the top three leaves, flag leaf (A), second leaf (B) and third leaf (C), of a slow-senescing NPT line (IR67966-44-2-3-2) and a fast-senescing NPT line (IR68552-100-1-2-2) from flowering to maturity. Total content of active cytokinins consist of Z, DHZ, ZR, DHZR, iP and iPR.

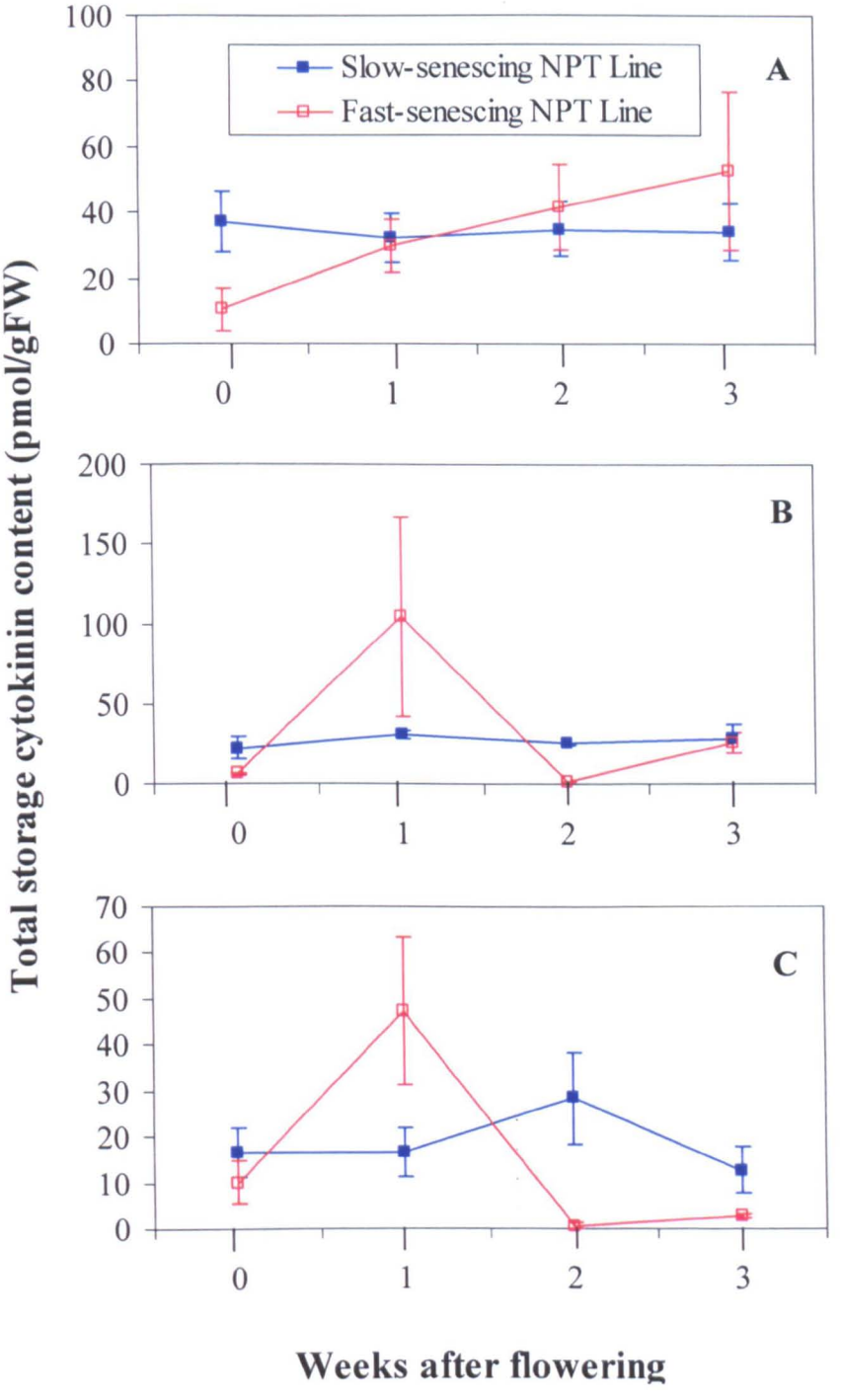

Figure 2.3. The total content of all storage cytokinins in the top three leaves, flag leaf (A), second leaf (B) and third leaf (C), of a slow-senescing NPT line (IR67966-44-2-3-2) and a fast-senescing NPT line (IR68552-100-1-2-2) from flowering to maturity. Total content of storage cytokinins consist of DHZOG, ZOG, ZROG and DHZROG.

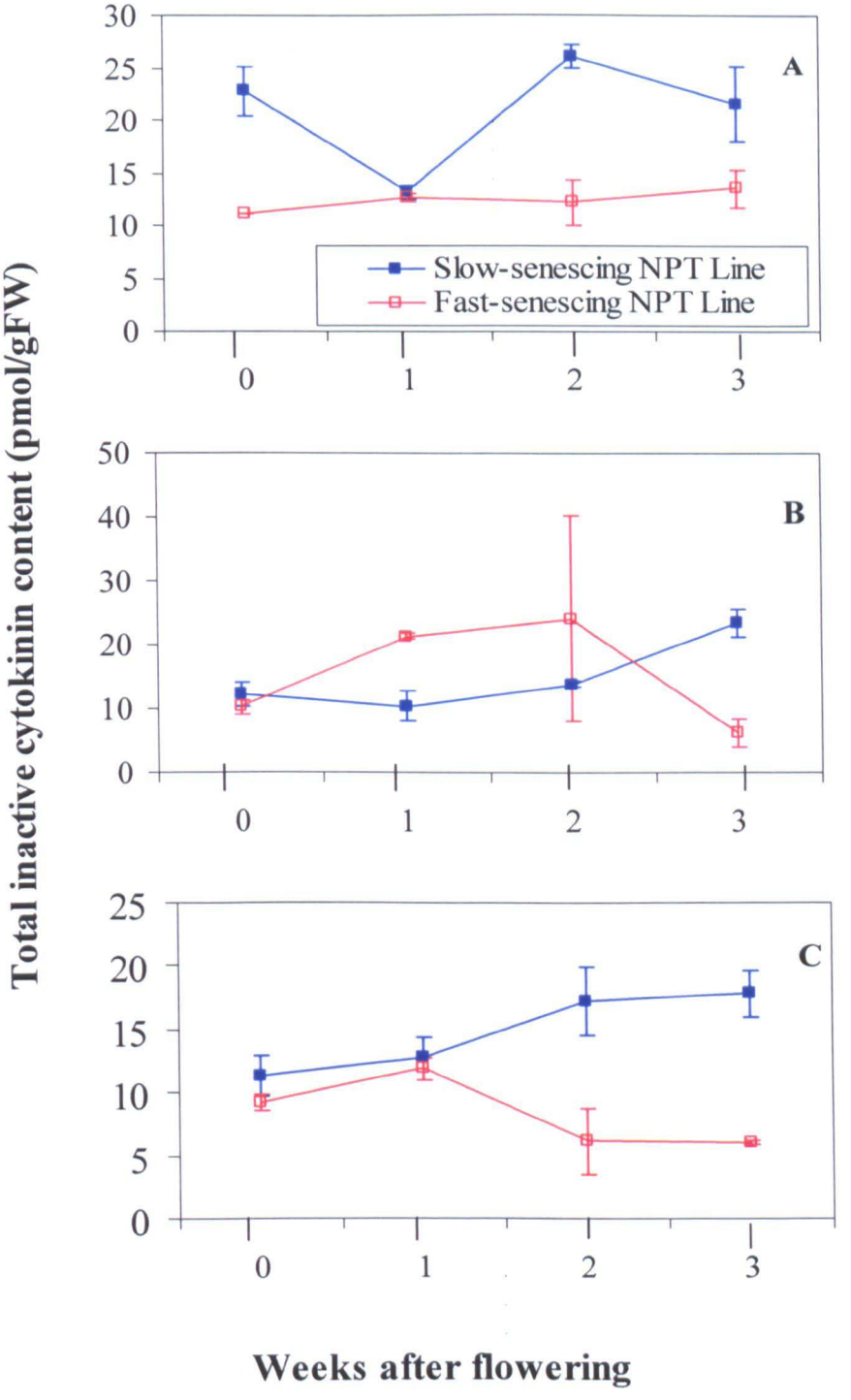

Figure 2.4. The total content of all inactive cytokinins in the top three leaves, flag leaf (A), second leaf (B) and third leaf (C), of a slow-senescing NPT line (IR67966-44-2-3-2) and a fast-senescing NPT line (IR68552-100-1-2-2) from flowering to maturity. Total content of storage cytokinins consist of Z7G, Z9G, DHZ7G, DHZ9G, iP7G and iP9G.

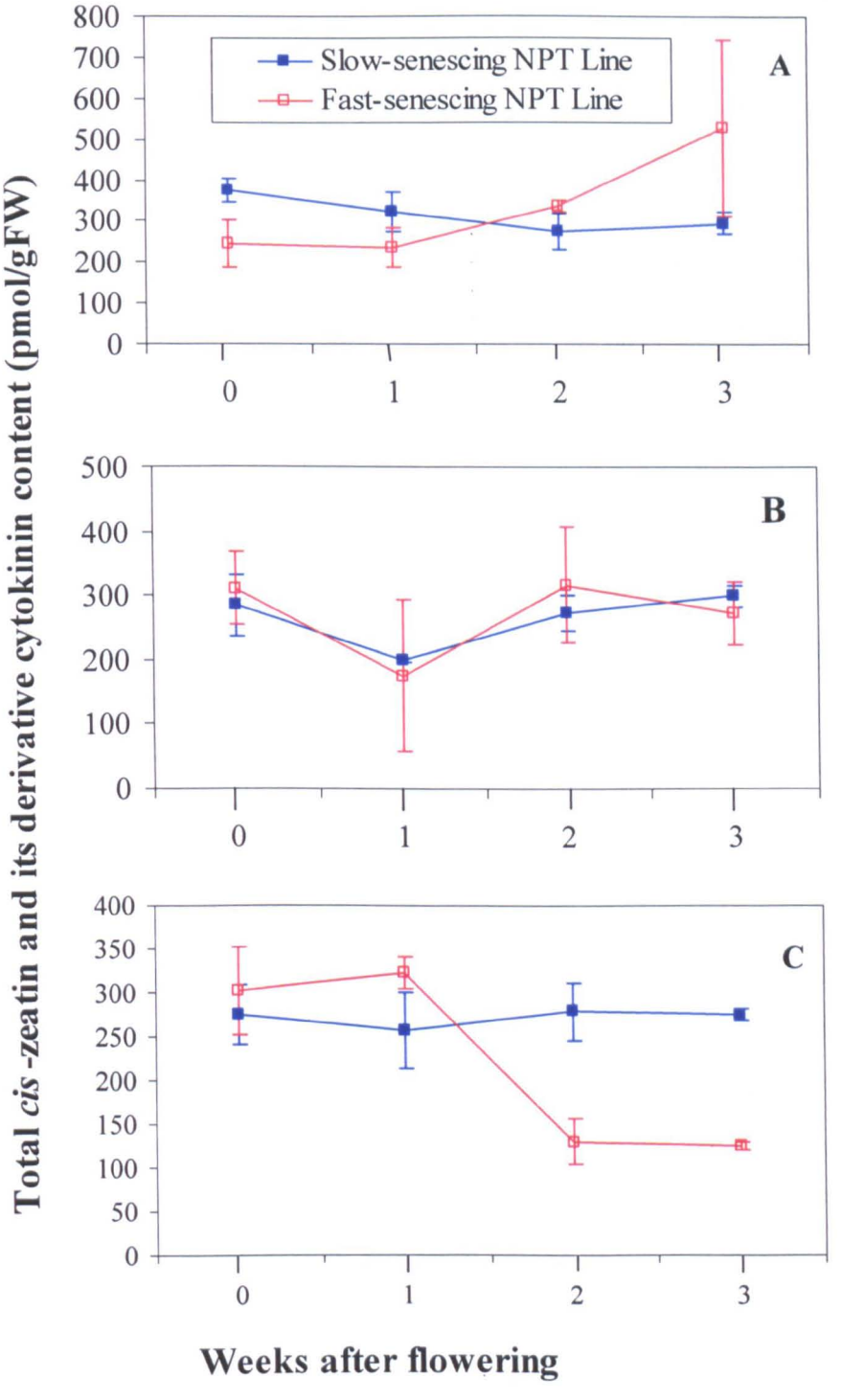

Figure 2.5. The total content of all cis-zeatin and its derivative cytokinins in the upper top leaves, flag leaf (A), second leaf (B) and third leaf (C), of a slow-senescing NPT line (IR67966-44-2-3-2) and a fast-senescing NPT line (IR68552-100-1-2-2) at flowering to maturity. Total content of *cis*-zeatin and its derivative cytokinins consist of *c*-Z, *c*-Z7G, *c*-Z9G, *c*-ZOG, *c*-ZROG and *c*-ZR.

The ANOVA analysis (Table 2.3) showed that there was no significant difference in the levels of four groups of CKs in the top three leaves during their aging between 0 to 3 WAF in both NPT lines. However, there was a significant difference in the levels of (a) active CKs in the second leaf of the slow-senescing NPT line and in the flag leaf and second leaf of the fast-senescing NPT line, (b) storage CKs and *cis*-zeatin and its derivatives in the third leaf of fast-senescing NPT lines and (c) inactive CKs in the flag leaf and the second leaf of the slow-senescing NPT line.

The changes in the levels of CKs in the top three leaves of a fast-senescing NPT line (IR68552-100-1-2-2) and a slow-senescing NPT line (IR67966-44-2-3-2) were observed at 0, 1, 2 and 3 WAF. The content of active CKs at the time of flowering was two-fold higher in the fast-senescing NPT line compared to slow senescing NPT line but it sharply declined with the leaf age in the flag leaf (Figure 2.2). There was no significant difference in the content of active CKs found in the second and third leaves at flowering between the two NPT lines. A progressive decrease in active CKs was associated with the progressing age of the flag leaf and the second leaf of the fast-senescing NPT line while opposite trend was found in slow-senescing NPT line. Interestingly, the level of active CKs was increasing with age of the third leaves of both NPT lines and this increase was more pronounced in the fast-senescing NPT line. The level of storage CKs remained at a steady level in the three leaves of the slow-senescing NPT line (Figure 2.3). However, the content of storage CKs increased in the flag leaf, increased and then sharply decreased in the second and third leaves of the fast-senescing NPT line. The two NPT lines differed in the profiles of inactive CKs. The content of inactive CKs remained steady in the flag leaf, increased then sharply decreased in the second and third leaves of the fast-senescing NPT line (Figure 2.4). However, the level of inactive CKs increased in all the three leaves of the slow-senescing NPT line. The *cis*-zeatin and its derivative CKs remained at a steady level in all the leaves of the slow-senescing NPT line (Figure 2.5). However, their content was gradually increased in the flag leaf and second leaf but decreased in the third leaves of the fast-senescing NPT line.

The ANOVA results (Table 2.4) showed that there was no significant difference in the levels of four groups of CKs in the top three leaves during their aging between 0 to 3 WAF in both NPT lines. However, there was a significant difference in the levels of (a) active CKs in the second leaf of the slow-senescing NPT line and in the flag leaf and second leaf of the fast-senescing NPT line, (b) storage CKs and *cis*-zeatin and its derivatives in the third leaf of fast-senescing NPT line and (c) inactive CKs in the flag leaf and the second leaf of the slow-senescing NPT line.

2.3.5 Relationship between the cytokinin levels and chlorophyll contents

The correlation coefficients between the chlorophyll content and the levels of four groups of CKs in the leaves of fast-senescing and slow-senescing NPT lines during grain filling are shown (Table 2.5). No significant correlation was observed between the chlorophyll content and the level of four groups of CKs in the top three leaves of both NPT lines. However, there was a significant correlation between the chlorophyll content and the level of a.) active CKs in the second leaf and inactive CKs in the third leaf of the slow-senescing NPT line, b.) inactive CKs and *cis*-Z and its derivatives in the flag leaf and active CKs in the third leaf of the fast-senescing NPT line.

Table 2.4. A single-factor analysis of variance of the total content of different cytokinins in the top three leaves of slow-senescing and fast-senescing NPT lines during grain filling.

NPT line	Type of leaf	Computed F value of the content of different CKs (pmol/gFW)			
		Active CKs	Inactive CKs	*Cis*-zeatin and its derivative CKs	Storage CKs
Slow-senescing NPT line (IR67966-44-2-3-2)	Flag leaf	0.92 ns	5.65 *	1.34 ns	0.05 ns
	Second leaf	5.20 *	6.27 *	2.37 ns	0.4 ns
	Third leaf	2.86 ns	2.16 ns	0.10 ns	1.03 ns
Fast-senescing NPT line (IR68552-100-1-2-2)	Flag leaf	4.24 *	0.32 ns	1.44 ns	1.53 ns
	Second leaf	4.27 *	1.07 ns	0.61 ns	2.36 ns
	Third leaf	2.01 ns	3.14 ns	13.04 **	6.84 **

Note: ns = not significant; * = significant at 0.10 level of probability (4.19 = tabulated F value for two-tailed analysis); **= significant at 0.05 level of probability (6.59 = tabulated F value for two-tailed analysis).

Table 2.5. Coefficients of correlation between chlorophyll contents and the levels of four groups of cytokinins in the top three leaves of a slow-senescing NPT line and a fast-senescing NPT line during grain filling.

NPT line	Group of cytokinins	Correlation coefficient		
		Flag leaf	Second leaf	Third leaf
Slow-senescing NPT line (IR67966-4-2-3-2)	Active cytokinins	-0.03 ns	-0.90 *	-0.70 ns
	Inactive cytokinins	-0.31 ns	-0.76 ns	-0.99 ***
	Cis-zeatin and its derivative cytokinins	0.57 ns	-0.57 ns	-0.43 ns
	Storage cytokinins	0.18 ns	-0.24 ns	-0.27 ns
Fast-senescing NPT line (IR68552-100-1-2-2)	Active cytokinins	0.63 ns	0.43 ns	-0.99 ***
	Inactive cytokinins	-0.91 *	0.21 ns	0.68 ns
	Cis-zeatin and its derivative cytokinins	-0.95 **	-0.23 ns	0.82 ns
	Storage cytokinins	-0.86 ns	0.29 ns	0.42 ns

ns = not significant; * = significant at 0.10 level of probability [r 0.10 (2-tailed), 2 df = 0.90]; ** = significant at 0.05 level of probability [r 0.05 (2-tailed), 2 df = 0.95]; *** = significant at 0.01 level of probability [r 0.01 (2-tailed), 2 df = 0.99]

2.4 Discussion

The present study shows the changes in chlorophyll content in the top three leaves of four NPT lines during the grain filling period. The flag leaf of the four NPT lines also remained green almost until maturity of the grains. The chlorophyll content of the top three leaves gradually decreased from flowering to maturity in the four NPT lines. A decrease in chlorophyll content occurred earlier in the third leaves followed by the second leaf and then the flag leaf in these four NPT lines which indicate that all these NPT lines showed a sequential mode of senescence. Two leaf senescence patterns were observed, namely, fast and slow senescence. IR68552-100-1-2-2 and IR69092-57-3 were fast-senescing NPT lines whereas IR67962-40-6-3-3 and IR76966-44-2-3-3 were slow-senescing NPT lines. Previous studies have shown that the flag leaves remain green almost until grain maturity (Saha *et al.*, 1986) and the chlorophyll content in the upper three leaves decreased during grain filling of other rice cultivars (Mondal and Choudhuri, 1984; Mae *et al.*, 1985; Park *et al.*, 1993; Soejima *et al.*, 1995). These studies reported only the sequential mode of senescence in different rice cultivars (Mae *et al.*, 1985; Park *et al.*, 1993; Soejima *et al.*, 1995). However, Mondal and Choudhuri (1984) found that Masuri, Kalojira and Pusa rice cultivars showed sequential mode of senescence but Jaya and Ratna cultivars showed a non-sequential mode of senescence wherein the younger flag leaf senesced earlier than the older second leaf from the top of the rice plant.

In the present study, the yield components and grain yield differ among the four NPT lines. The results indicate that the total number of spikelets per m^2 and the number of spikelets per panicle in the two slow-senescing NPT lines, IR67962-40-6-3-3 and IR76966-44-2-3-3, were higher than in the two fast-senescing NPT lines, IR68552-100-1-2-2 and IR69092-57-3. However, the higher the total number of spikelets did not show that it will lead to higher yield when there is low grain filling percentage. Yang *et al.*, (2002a) also reported that the total number of spikelets did not translate into greater yield because of their low spikelet filling percentage in the japonica/indica hybrid rice.

In this study, the grain weight was lower in the slow-senescing NPT lines compared to the fast-senescing NPT lines. Yang *et al.*, (1997) and Zhu *et al.*, (1997) reported that delayed senescence retards remobilization and can lead to reduced grain weight of hybrid rice.

A number of studies have reported that delayed leaf senescence increased grain filling and yield after application of kinetin in rice (Ray and Choudhuri, 1981) and after nitrogen fertilizer in oats (Peltonen-Sainio 1997). Delayed leaf senescence has also increased the yields in non-senescent sorghum hybrids (Ambler *et al.*, 1992).

In the present study, the grain filling percentage was higher in the two fast-senescing NPT lines than the two slow-senescing lines which suggests that faster leaf senescence result to higher grain filling percentage. This result was consistent with the results of Park *et al.*, (1993) wherein rapid senescing rice cultivars showed higher grain filling percentage compared to the slow-senescing cultivars. Park *et al.*, (1993) reported that the percentage of filled grains of the slow, moderately slow, medium and rapid senescing rice cultivars was 72%, 74.8%, 76.4& and 81.6%, respectively. Yang *et al.*, (2002b) reported that water stress enhanced leaf senescence in rice, accelerated starch remobilization, shortened grain filling period and increased grain filling rate.

Our results showed that the number of empty spikelets was higher than the number of half-filled spikelets in IR69092-57-3, but the opposite trend was observed in the other 3 NPT lines. This result indicate that either the number of empty spikelets or the number of half-filled spikelets was affected the spikelet filling percentage. Lu *et al.*, (1994) also reported that low spikelet percentage of japonica/indica hybrids was mainly due to the high number of empty or sterile spikelets. But Yang *et al.*, (2002a) reported that the high number of half-filled spikelets was the cause of poor grain filling percentage in japonica/indica hybrid rice. The poor grain filling of this hybrid was a result of poor translocation and

partitioning of assimilates into the grains rather than limited biomass production of source assimilates.

Previous studies have shown that the rate of leaf senescence is positively correlated with grain filling percentage in rice (Park *et al.*, 1993; Yang *et al.*, 2002a) but negatively correlated with grain yield in rice (Park, 1991) and sorghum (Borrell *et al.*, 2000). In the present study, the rates of senescence in the top three leaves were positively correlated with grain filling percentage of the four NPT lines which indicate that the faster the senescence in the top three leaves, the higher the grain filling percentage. However, the grain yield was positively correlated only with the rate of senescence in the flag leaf which indicate the importance of the flag leaf during the grain filling period.

In previous studies, Takagi *et al.*, (1985) detected only five CKs in the rice shoots using deuterium labelled internal standards and selected ion monitoring whereas Saha *et al.*, (1986) detected only glucoside-like activity and ZR-like activity in the upper four leaves of rice using soybean callus bioassay. In this study, 22 CKs were identified in the top three leaves of fast and slow senescing NPT lines using deuterium-labelled CK internal standards and HPLC/MS. The 22 CKs were placed into four functionally different groups of CKs, namely, active CKs (CK bases and ribosides), storage CKs (CK *O*-glucosides), inactive CKs (CK-7-*N*-glucosides and CK-9-*N*-glucosides) and *cis* derivatives of zeatin.

In previous work, only Z and ZR were the active CKs observed in the rice leaves (Oritani and Yoshida, 1973). A rapid fall in Z levels took place with decrease in chlorophyll content but ZR and bound Z contents did not change. Yue *et al.*, (1996) found that the ZR decreased rapidly in the flag leaf after flowering of wheat plants. Saha *et al.*, (1986) observed that the flag leaf maintained higher Z and ZR-like CK activity than the other upper leaves of rice. The high CK-like activity in the top four leaves during grain filling indicates that these leaves play a major role in this process. This study shows that the differences in the dynamics of CKs between fast-senescing and slow-senescing NPT lines in the flag leaf are

more important for control of senescence than the differences in actual CK levels. The older second and third leaves are much less active in production of assimilates and they are apparently less responsive to changes in CKs content. Different leaves of slow-senescing and fast-senescing NPT lines showed very different dynamics of active CKs between 0 and 3 WAF. In the slow-senescing NPT line, the levels of active CKs in the flag leaf and third leaf were not significantly changed from flowering to maturity which indicate that the active CKs are relatively stable. Relatively stable level of active CKs in the flag leaf, which is the main source of photoassimilates, may delay its senescence in the slow-senescing NPT line. A progressive increase in levels of active CKs in the slow-senescing NPT line was positively correlated with gradual accumulation of inactive CKs. These results indicate that there is a higher rate of biosynthesis and/or import of active CKs as well as their turn-over which may be favorable for prolongation of the period of photosynthetic activity and delay of leaf senescence.

There was a dramatic decrease in active CKs in the flag leaf and second leaf of the fast-senescing NPT line but the levels of active CKs in these leaves of the slow-senescing NPT line showed an opposite trend. Interestingly, the level of active CKs was gradually increasing with age of the third leaves of the fast-senescing NPT line which may be due to the release of CKs from tRNAs, which are, together with proteins, degraded in old leaves and reutilised after their translocation to grains. The increase in content of active CKs in the third leaf of the fast-senescing NPT line was inversely proportional to the level of storage CKs and inactive CKs indicating that the increase of level of active CKs in this leaf could be a result of reduced inactivation of CKs by *O*- and *N*-glucosylation. Glucosylation of CKs in leaves occurs when the CKs are no longer needed for active growth, hence, the storage CKs can be converted to active CKs which can be used at later phases of plant development (Van Staden and Davey, 1979; Saha *et al.*, 1984).

The storage CKs (CK-*O*-glucosides) fluctuate as the leaf senesces (Van Staden 1976; Parker and Letham., 1973; Saha *et al.*, 1984; Van Staden 1983). Saha *et al.*,

(1986) observed that the total CK glucoside-like activity in the upper four leaves of rice decreased from flowering to maturity. In this study, the increased level of storage CKs in the flag leaf of the fast-senescing NPT line indicates that active CKs are gradually converted to their corresponding storage forms. However, the level of storage CKs did not significantly change during development of all analysed leaves of the slow-senescing NPT line which indicate that the storage CKs are stable in the slow-senescing NPT line.

Enzymatic *trans-cis* conversions of Z by *cis-trans* isomerase have not been reported in rice so far. The levels of *cis*-Z derivatives in different leaves of the slow-senescing NPT line are not significantly changed between 0 and 3 WAF. However, the negative correlation between the level of active CKs and derivatives of *cis*-Z in the three leaves of the fast-senescing NPT line indicates their possible involvement in regulation of active CK levels in these leaves. Such regulation did not occur in different leaves of slow senescing NPT line where the content of *cis*-Z derivatives remained relatively constant during aging of all leaves of slow-senescing NPT line.

Statistical analysis showed that most differences in CK levels were not significant. Nevertheless, interesting trends were observed. There was no significant change in active CKs, *cis*-zeatin derivatives and storage CKs in different leaves of slow-senescing NPT line during grain filling period. This supports the conclusion that the content of these CKs in the slow-senescing NPT line is relatively stable. On the other hand, fast changes in active CK levels in the flag leaf and second leaf of fast-senescing NPT line are significant supporting the idea that the changes in dynamics of CKs in leaves of this line are decisive for fast senescence.

Previous studies have shown that there was no significant correlation between the chlorophyll content and the total concentration of cytokinins, the total flux of CKs per plant and the total flux per unit leaf area in the flag leaves and second leaves but not in the third leaves (Soejima *et al.*, 1995). These results indicated that delayed senescence in the flag leaves and second leaves of the slow-senescing rice

cultivar was not due to the presence of large amounts of xylem-sap CKs but may be due to other unidentified factors. In this study, there was also no significant correlation between the chlorophyll content and the four groups of CKs in the flag leaves, second leaves and third leaves of slow-senescing and fast-senescing NPT lines except in the *cis*-Z and its derivative CKs in the flag leaf of the fast-senescing NPT line and in the inactive CKs in the third leaf of the slow-senescing NPT line.

This study shows that the sudden degradation of photosynthetic apparatus and re-utilization of metabolites by developing seeds is the scheme used by NPT rice for more efficient supply of photoassimilates to the developing seeds. The faster rate of leaf senescence resulted in a higher grain filling percentage. It is not the actual content of CKs in the flag leaf but the changes in their dynamics which are decisive for fast senescence. The levels of active, storage and *cis*-zeatin derivative CKs are relatively stable in the slow-senescing NPT line. There are strong indications that higher rate of biosynthesis and/or import of active CKs as well as their high turn-over may be favourable for prolongation of the period of photosynthetic activity and delay of leaf senescence in leaves of slow senescing NPT line. An investigation of the changes in the cytokinin levels in the roots and grains of the fast and slow senescing NPT lines remain to be pursued.

Chapter 3. Expression of the SAG12-*ipt* gene and cytokinin levels in the leaves of transgenic New Plant type rice

3.1. Introduction

Leaf senescence is the final stage of leaf development wherein the nutrients are distributed to newly developing leaves or grains (Nooden, 1988). Although leaf senescence plays a role in the evolutionary fitness of plants, it is not always welcomed with respect to higher plant production in agriculture (Lin *et al.*, 2002). Leaf senescence devalues ornamental plants and foliar vegetables during transportation and storage. Leaf senescence also contributes to limitation of yield in some monocarpic crops such as soybean (Nooden, 1988). Since the top three leaves are the main sources of photosynthates during grain filling (Yoshida, 1971), prolonging the photosynthetic life span of these leaves by delaying senescence may improve rice grain filling.

Leaf senescence can be delayed by exogenous application or genetic manipulation of cytokinins. Cytokinins (CKs) are plant hormones which naturally prevent senescence and maintain photosynthetic activity in leaves (Sharma *et al.*, 2002). Spraying rice plants with CKs had a pronounced effect on grain-filling and increased the yield which was possibly due to prolonged photosynthetic life span (Ray and Choudhuri, 1981). Exogenous application of CKs in rice plants increased grain filling and yield (Ray and Choudhuri, 1981; Oritani and Murasaki, 1990). Ray and Choudhuri (1981) sprayed 100μg/ml aqueous solutions of kinetin on Jaya rice cultivar when the plants were entering the reproductive stage. Kinetin increased grain filling and harvest index, which represents grain yield, of the plants which may be due to increasing leaf longevity. The control plants had 80.22% spikelet fertility and harvest index of 56.08 whereas the plants sprayed with kinetin had 89.54% spikelet fertility and harvest index of 61.66. Oritani and Murasaki (1990) also sprayed rice plants with the synthetic CKs, called TG-19, which were soluble in water and easily absorbed into and translocated on plants. The three uppermost leaves maintained a high level of assimilation activity even

on the 40th day after flowering in the rice plants sprayed with TG-19. However, the control plants declined rapidly in its assimilation activity from the 20th day after flowering. The control plants had a grain filling percentage of 86% and 454.2 kg/992m^2 whereas the plants sprayed with TG-19 had 91.2% grain filling percentage and a yield of 509.1 kg/992m^2. Hence, spraying the leaves with TG-19 increased the ripening percentage by approximately 5% which resulted in a 12% increase in yield. However, large scale application of cytokinins in the field is a costly operation and there is a risk of side effects such as inhibition of root development when CKs are splashed down into the rhizosphere (Kaminek *et al.*, 2003). To augment this problem, genetic manipulation of CKs levels using the isopentenyl transferase (*ipt*) gene is a much more sophisticated method and potentially efficient in delaying leaf senescence.

The cytokinin biosynthetic *ipt* gene, from *Agrobacterium tumefaciens*, encodes for the isopentenyl transferase enzyme which catalyzes the conversion of isopentenyl pyrophosphate and adenosine monophosphate (AMP) to isopentenyl AMP (Akiyoshi *et al.*, 1984; Barry *et al.*, 1984). Isopentenyl AMP is subsequently metabolized to isopentenyl-adenosine and isopentenyl-adenine. Hydroxylation of these latter compounds results in zeatin-riboside and zeatin, respectively, and serves as a precursor of the other active cytokinins in plants. CKs are one of the major classes of plant hormones which influence plant growth and differentiation, affect various plant growth and developmental processes in whole plants such as release of axillary buds from dormancy (Wickson and Thimann, 1958), inhibition of root formation and branching (Wightman *et al.*, 1980), closure of stomata, control of chloroplast development (Parthier, 1979), promotion or inhibition of flowering and fruit set in some species (Bernier, 1988; Carlson *et al.*, 1987) and retardation of leaf senescence (Richmond and Lang, 1957).

Overexpression of the *ipt* gene driven by constitutive promoters results in shooty teratomas and inhibits root formation and regeneration of whole plants (Smigocki and Owens, 1988). Since constitutive expression of the *ipt* gene completely inhibits root formation in the transgenic plants, this problem was attempted to be

solved by fusing the *ipt* gene to heat-regulated or wound-inducible promoters (Ainley *et al.*, 1983; Medford *et al.*, 1989; Schmulling *et al.*, 1989; Smart *et al.*, 1991; Smigocki *et al.*,1991; Smigocki *et al.*,1995; Van Loven *et al.*,1993), tissue-specific promoters (Beinsberger *et al.*, *et al.*,1992; Li *et al.*, 1992a; Martineau *et al.*, 1994) or CaMV 35S promoter disrupted by Ac transposon (Estruch *et al.*, 1991). The endogenous CK levels have increased in *Nicotiana*, *Arabidopsis* and *Petunia* plants expressing the *ipt* gene fused to constitutive promoters (Medford *et al.*, 1989; Smigocki and Owens, 1988) or heat-inducible promoters (Medford *et al.*, 1989; Schmulling *et al.*, 1989; Smigocki, 1991). However, these transgenic plants are morphologically and developmentally abnormal (Mok and Mok, 1994). The expression of the *ipt* gene results in delayed leaf senescence, shorter transgenic plants, increased lateral budding and viviparous leaves, less developed roots and abnormal flower development.

The expression of the *ipt* gene driven by promoters regulated by environmental and developmental factors produced transgenic plants which exhibited altered morphologies. To circumvent these undesirable effects of overexpression of the *ipt* gene, Gan and Amasino (1995) devised a strategy based on autoregulated cytokinin production using the *ipt* gene driven by a senescence-specific promoter *SAG12* from *Arabidopsis thaliana.* At the start of leaf senescence, the *SAG12* promoter should activate the expression of the *ipt* gene and increase the cytokinin content to a level which prevents the leaf from senescing. When senescence is prevented, it attenuates the expression of the *SAG12* promoter to prevent overexpression of CKs. Since CK production is triggered only at the start of leaf senescence due to the regulation of the *ipt* gene by the *SAG12* promoter then the plant grows normally.

Previous reports have shown that SAG12-*ipt* gene was successfully introduced into tobacco (Gan and Amasino, 1995), cauliflower (Nguyen *et al.*, 1998), lettuce (McCabe *et al.*, 1998), broccoli (Chen *et al.*, 2001; Gapper *et al.*, 2002), wheat (Daskalova *et al.*, 2002), and rice (Fu *et al.*, 1998; Cao, 2001 and Lin *et al.*, 2002). Leaf senescence was delayed in the leaves of transgenic lettuce plants and the

florets of transgenic broccoli plants but did not show serious morphological abnormality (Chen *et al.*, 2001; McCabe *et al.*, 2001).

Transgenic SAG12-*ipt* tobacco plants showed delayed leaf senescence, increased yield and biomass without having any growth abnormalities (Gan and Amasino, 1995). Transgenic Millin, Minghui and Minghui 63 rice cultivars containing the SAG12-*ipt* gene also showed delayed leaf senescence and increased grain filling without any plant abnormalities (Cao, 2001; Lin *et al.*, 2002). All the transgenic T_0 wheat plants expressing the *ipt* gene under the control of SAG12 promoter have exhibited signs of male sterility (Daskalova *et al.*, 2002). Although there was no morphological abnormality, the onset of anthesis was delayed for at least a week. The senescence of leaves after anthesis was delayed between one to two weeks. There was no increase in grain filling but there was an increase in grain weight ranging from 5% to 30% for the different wheat lines.

In this study, *gusA* and *ipt* genes under the control of the senescence-specific SAG12 promoter were introduced into rice using particle bombardment. The integration, expression and inheritance of *gusA* and *ipt* genes in the transgenic plants were determined. The grain filling percentage and cytokinin levels in the leaves of the transgenic SAG12-*ipt* plants were also investigated.

3.2. Materials and methods

3.2.1 Plant Material

Three tropical japonica New Plant Type (NPT) rice lines (IR65600-42-5-2, IR66160-121-4-5-3 and IR68552-100-1-2-2) and two indica rice varieties (IR72 and IR64) were used in this study.

3.2.2 Plasmid Constructs

Three plasmids constructs, namely, pSG506, pSG516 and pTRA132 were used in biolistic transformation of rice (Figure 3.1, 3.2 and 3.3, respectively). pTRA132 containing the *hpt* gene was co-transformed with either pSG506 containing the *gusA* gene or pSG516 containing the *ipt* gene.

3.2.3 Callus induction, biolistic transformation, selection and plant regeneration

Mature rice seeds were dehulled, surface-sterilized with 70% ethanol for 2 minutes and rinsed three times with sterile distilled water. The seeds were sterilized with 2.5 % sodium hypochlorite for 35 minutes, shaken at 120 revolution per minute (rpm) and rinsed thoroughly with sterile distilled water. The sterilised seeds were transferred on sterile filter papers to absorb the excess water. Ten seeds were inoculated per petri dish (100mm x 15 mm) containing the callus induction medium (MS salts with 30g/l maltose, 2 mg/l 2,4-D and 0.8% agarose. The seeds were kept at 25°C in the dark for 6 weeks for callus induction. About 75-100 pieces of embryogenic calli were selected and placed at the center of a petri dish containing the callus induction media. Gold particles (1 μm) were coated with pTRA132 containing CaMV35S-*hpt* gene together with either pSG506 containing SAG12-*gusA* gene or pSG516 containing SAG12-*ipt* gene. The embryogenic calli were bombarded with a helium-driven particle delivery system, Model PDS-1000/He Biolistic (BioRad). Rupture disk pressure ratings were 1100, 1350 or 1500 pounds per square inch (psi) in each bombardment. The bombarded calli were kept at 25°C in the dark and subcultured every 2 weeks on fresh selection medium (callus induction media with 50 mg/L hygromycin B). The calli which survived the selection were transferred to a plant regeneration medium (N6 salt with 1 mg/l NAA, 8 mg/l kinetin, 30 g/l maltose) containing 50mg/l hygromycin B and kept at 28°C in the light. When plantlets were formed, they were transferred onto the rooting media (N6 medium with 40 g/l sucrose, 0.7% agarose) with 50mg/l hygromycin B. When more roots were formed, the plantlets

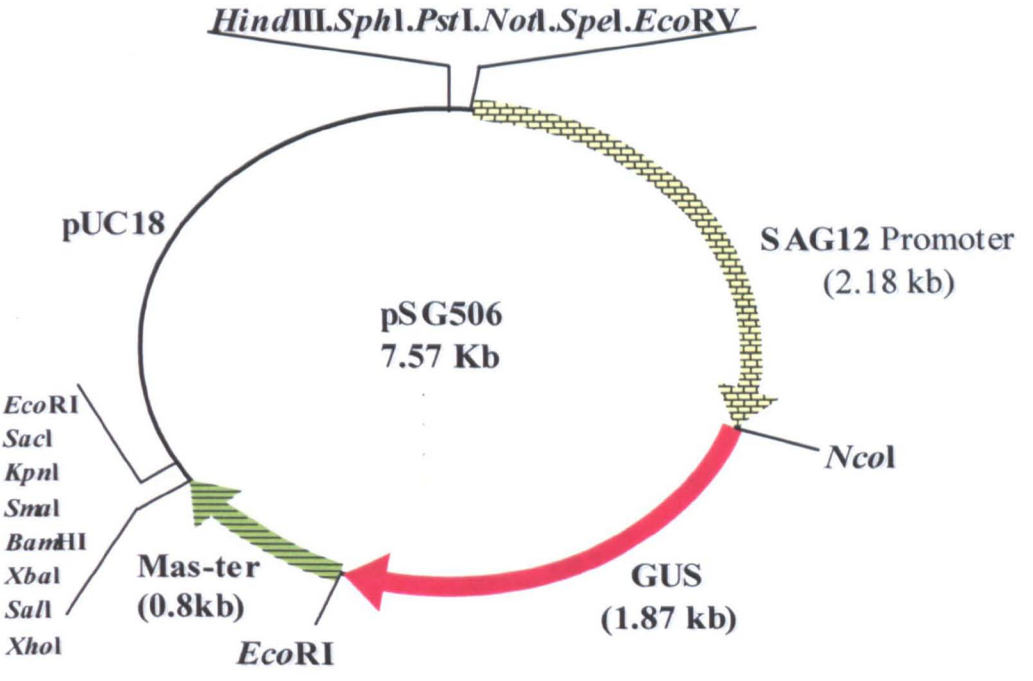

Figure 3.1 Schematic map of pSG506 harbouring SAG12-*gusA* gene.

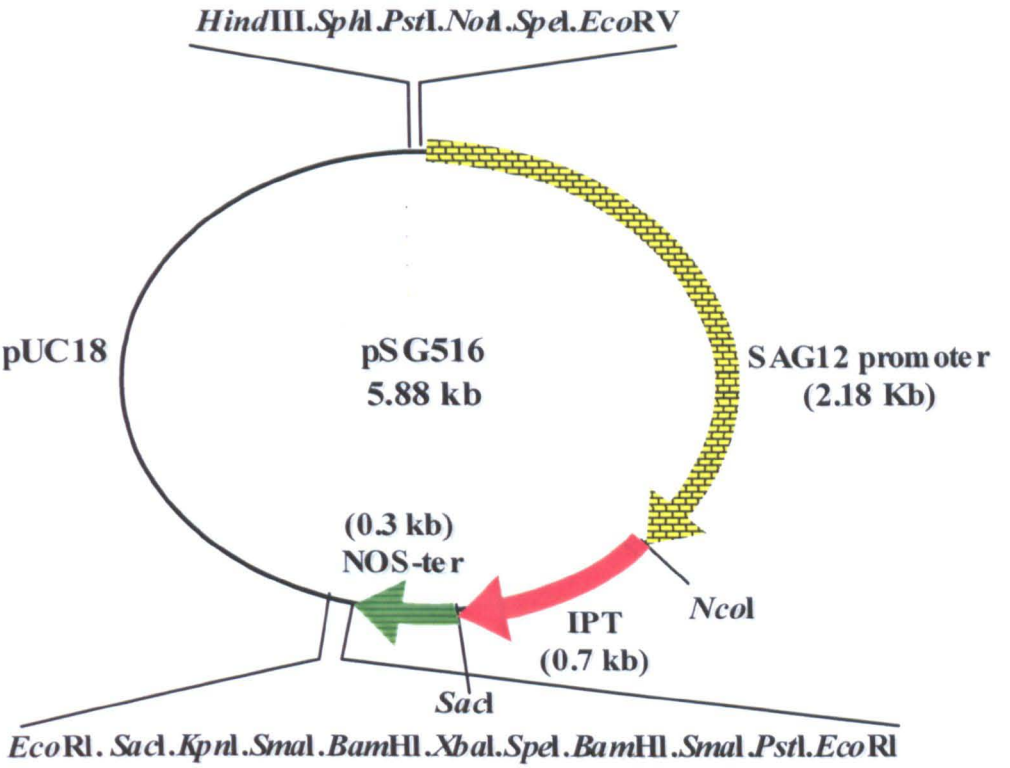

Figure 3.2 Schematic map of pSG516 harbouring the SAG12-*ipt* gene.

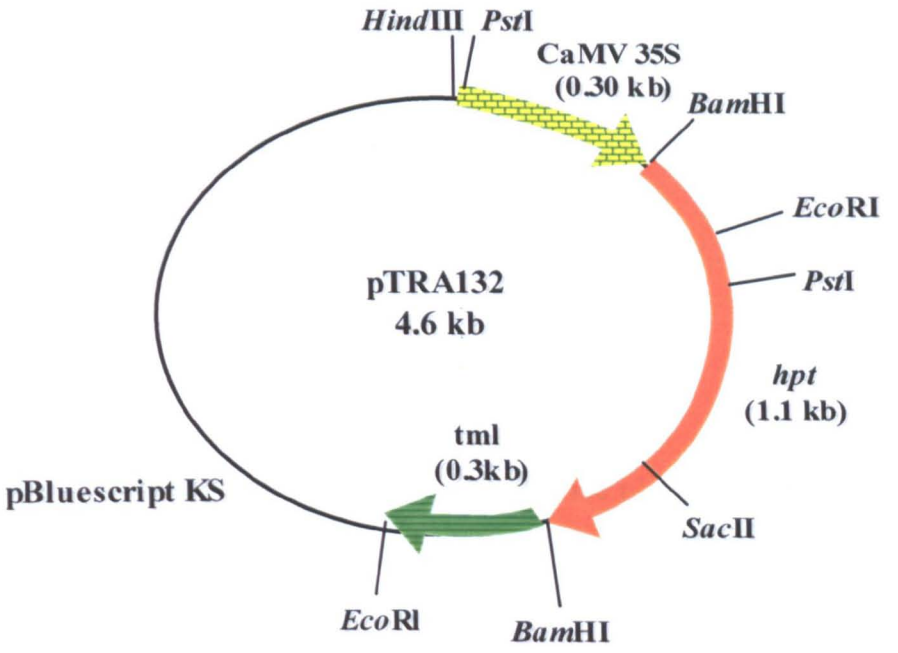

Figure 3.3 Schematic map of pTRA132 harbouring the CaMV35S-*hpt* gene.

were grown in Yoshida's culture solution for 3 weeks then transferred into individual pots with autoclaved soil and grown until plant maturity in the contained greenhouse (28-32°C:22-25°C; 12 hours light:12 hours dark; 70-80% relative humidity).

3.2.4 Molecular analysis of the transgenic rice plants

3.2.4.1 Extraction of DNA

a. Miniscale Extraction of DNA

DNA was extracted from putatively transformed rice plants and their progeny by the method of Zheng *et al.*, (1995). Fresh leaf samples, about 2-3 cm long, were collected and placed in pre-labelled tubes and dipped in liquid nitrogen. The leaf tissue was cut into small pieces and placed in a well of Spot Test Plate (Thomas Scientific). Four hundred µl of DNA extraction buffer (50 mM Tris, pH 8.0, 25 mM EDTA, pH 8.0, 300 mM NaCl, 1% SDS) was added to it. The tissue was ground with a glass rod and the extract was transferred in a 1.5 ml tube. Four hundred µl of chloroform was added to the tube containing the extract. The tube was mixed well then centrifuged for 30 seconds. The supernatant was transferred to another 1.5 ml tube. Eight hundred µl of absolute ethanol was mixed with the supernatant. The mixture was placed at -20°C for at least 30 minutes and centrifuged for 3 minutes at 12,000 rpm. The supernatant was discarded. The DNA pellet was washed with 70% ethanol and air-dried. The DNA pellet was resuspended in 50 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM Na_2EDTA) and stored in -20°C until used in PCR analysis.

b. Large Scale Extraction of Genomic DNA

DNA was extracted from the untransformed and transformed rice plants as described by Dellaporta *et al.*, (1983). The leaves (5 g) of 30-45 day old plants were collected and ground to a fine powder in mortar with the aid of a pestle and a

liquid nitrogen. Preheated extraction buffer [100mM Tris, pH 8, 20mM Na_2EDTA, 500mM NaCl, 1.25% SDS (w/v), 0.38% sodium bisulfite (w/v)] was added to the powdered tissue and incubated at 65°C for 15 minutes. Five ml of 5M potassium acetate was added and the mixture was placed in ice and shaken for 20 minutes. The sample was centrifuged at 3000 rpm for 20 minutes at +4°C. The supernatant was filtered using a miracloth and 2/3 volume of cold isopropanol was added to the supernatant. The supernatant was incubated at -20°C overnight and centrifuged at 2500 rpm at +4°C for 20 minutes. The supernatant was discarded and the DNA pellet was washed with 70% ethanol. The pellet was air-dried and resuspended in 3000 µl of TE buffer. Ten µl of RNAse (10mg/ml) was added before incubation at 37°C for 15 minutes. A tenth of a volume of 3M sodium acetate, pH 5.2 and 2 volumes of cold absolute ethanol were added to the mixture and shaken gently. The mixture was incubated at -20°C overnight and centrifuged at 2500 rpm for 15 minutes at +4°C. The supernatant was discarded and the DNA pellet was washed with 70% ethanol, air-dried, resuspended in 50-300 µl TE buffer and stored at -20°C.

3.2.4.2 Polymerase Chain Reaction

a. Design of Primers for Polymerase Chain Reaction (PCR)

Primers were designed to have more than least 50% GC content and not to have any complementary sequence between the primers. Table 3.1 shows the sequence of the forward and reverse primers designed used in the PCR analysis of the putative transformed rice plants and their progeny.

b.Controls for PCR Reactions

Four controls, included in every PCR reaction, served as a guide to the correct interpretation of the results. The controls were the following: minus DNA control, negative control, positive control and reconstructed positive control.

Table 3.1. The sequence of the primers used in PCR analysis of the *gusA, ipt* and *hpt* genes in the transgenic rice plants.

Gene/Locus	**Position of primer**	**Primer sequence**	**Expected size of the PCR product (kb)**
ipt	Forward	5' TTGCCCAGCAGACAGGGCTT 3'	0.7 kb
ipt	Reverse	5' CCGAACGGATGACCTTCGAATC 3'	
hpt	Forward	5' AGAATCTCG TGCTTTCAGCTTCGA 3'	0.7 kb
hpt	Reverse	5'TCAAGACCAATGCGGAGCATATAC3'	
gusA	Forward	5'CGATCAGTTCGCCGATGCAGATATT3'	1.1 kb
gusA	Reverse	5'ATATCCAGCCATGCACACTGATACT3'	
RG 100	Forward	5' GCTGGACGTGCCAAAGAGAG3'	0.9 kb
RG 100	Reverse	5' CGAACCACAGCCACAGCATG3'	

i. Minus DNA control uses water instead of any plant or plasmid DNA, hence,no PCR product is obtained.

ii. Negative control uses the DNA from the untransformed rice plant. Using the RG100 primers for rice locus RG100 found on chromosome 3 (Causse *et al.*, 1994), the expected size of the PCR product for the DNA from the untransformed rice plant is 0.9kb (Table3.1).

iii. Positive control uses the DNA from a plasmid DNA containing the gene of interest. The expected size of the PCR product for *gusA, hpt* and *ipt* genes are shown in Table3.1.

iv. Reconstructed positive control uses the DNA from the untransformed rice plant mixed with the plasmid DNA at a relative concentration to give one plasmid copy per haploid genome. Two PCR products are obtained, namely, 0.9 kb band for the DNA from the rice plant and the expected size of PCR product for the plasmid DNA containing the gene of interest shown in Table 3.1.

d. PCR analysis

The PCR reaction mixture was prepared using 2.5 µl 10x PCR buffer (10mM Tris-HCl, pH 8.4, 50mM KCl and 1.5mM $MgCl_2$), 3.2 µl 1 mM dNTPs, 2.5 µl 15 mM $MgCl_2$, 1 µl of 60 ng/µl of *ipt, hpt* or *gusA* forward primer, 1 µl of 60 ng/µl of *ipt, hpt* or *gusA* reverse primer, 10.3 µl distilled water, 0.5 µl of *Taq* polymerase and 2 µl of DNA. After initial denaturation at 95°C for 5 minutes, 35 cycles of 94°C for 1 minute, 60°C for 1 minute and 72°C for 1.5 minutes were carried out followed by final extension step 72°C for 7 minutes using PCR thermocycler. The PCR products were electrophoresed on 1% agarose gel.

3.2.4.3 Southern Blot

a. Controls for Southern Blot Analysis

Two controls were included in every DNA gel blot to serve as a guide in determining the presence and copy number of gene. The controls were the negative control and the positive control.

i. Negative Control

This control signified the absence of the gene of interest in the plant analyzed. The DNA from untransformed rice was used due to the absence of the gene. No band was expected unless a homologous sequence is present in the rice genome.

ii. Positive control

This control was for the estimation of the copy number of the gene of interest in transformed rice. One, five or ten copies of the gene of interest was mixed with 5.5 μg of untransformed rice DNA.

b. Southern Blot Analysis

Six μg of DNA from plants was digested with *EcoRI* and *NcoI* for the *gusA*-positive plants and *Spe I* for the *ipt*-positive plants. The undigested DNA of untransformed plant was mixed with 1, 5 and 10 copies of the *gusA* gene, *hpt* gene or SAG12-*ipt*-nos transgene. The undigested and digested DNA from untransformed and transformed plants, *Hind* III DNA marker, 1, 5 and 10 copies of the *hpt* gene, *gusA* ,SAG12-*ipt*-nos genes were loaded in 0.8% ethidium bromide-stained agarose gel. The gel was electrophoresed at 30 volts overnight in 1X TAE buffer. The gel was gently shaken in 0.25 M HCl for 30 minutes then in 0.4 M NaOH. The DNA was transferred to nylon membrane (Hybond N+, Amersham). The membrane was prehybridized with denatured salmon sperm DNA in a hybridization buffer at 65°C for 4 hours with gentle shaking. The DNA blots were hybridized with [^{32}P]- labelled 3.18 kb *SAG12-ipt-nos* probe or 1.87 kb

gusA probe at 65°C overnight. The probe DNA was labelled by random hexamer priming with Klenow fragment of DNA *pol I* (Amersham) and [alpha-^{32}P] dCTP (Amersham), based on the manufacturer's instruction. The membrane was washed stringently with 2x SSC (3M NaCl, 0.3M sodium citrate) and wrapped in saran wrap. The filters were analyzed by autoradiography using X-OMAT AR 5 film (Kodak) at -80°C. The film was developed by placing in a developer for 5 minutes, water for 1 minute and fixer for 3 minutes.

The membrane was stripped to remove the previous probe then washed with 2x SSC. The membrane was hybridized with [^{32}P]-labelled *hpt* probe, 1.1 kb *Bam* HI fragment of pTRA151, at 65 °C overnight. The *hpt* probe DNA was labelled by random hexamer priming with Klenow fragment of DNA *pol I* (Amersham) and [alpha-^{32}P] dCTP (Amersham), based on the manufacturer's instruction. The membrane was washed stringently with 2x SSC (3M NaCl, 0.3M sodium citrate) and wrapped in saran wrap. The filters were analyzed by autoradiography using X-OMAT AR 5 film (Kodak) at -80 °C.

3.2.4.4 RNA extraction

The total RNA was extracted from different types of leaves, namely, non-senescing, senescing and senesced leaves of untransformed and transformed rice plants using the SV Total RNA Isolation System (Promega). The leaf samples were ground into fine powder using mortar, pestle and liquid nitrogen. The powdered sample was placed in an eppendorf tube containing 175 µl of SV RNA lysis buffer. The tube was shaken to mix the sample with the lysis buffer then 350ul of SV RNA dilution buffer was added. The tube was inverted 3-4 times and centrifuged for 10 minutes at 12,000 x *g*. The cleared lysate was placed into a fresh eppendorf tube. The tube was mixed after adding 200 µl of 95% ethanol to the cleared lysate. The mixture was transferred to a spin basket assembly and centrifuged for 1 minute. About 600 µl of SV RNA wash solution was added then centrifuged for 1 minute and the eluate was discarded. About 50 µl of DNAse was added to the membrane and incubated at room temperature for 15 minutes. The

DNAse will degrade any DNA in the total RNA. After adding 200µl of SV DNase stop solution, the tube was centrifuged for 1 minute. After adding 600µl of SV RNA wash solution, the tube was centrifuged for 1 minute then emptied. After adding 250µl of SV RNA wash solution, the tube was centrifuged for 2 minutes. The liquid was transferred from the spin basket to the elution tube. After adding 100µl of nuclease-free water to the membrane, the tube was centrifuged for 1 minute to elute the RNA. The RNA sample was stored at -80°C.

3.2.4.5 cDNA synthesis using a Powerscript system

The cDNA primers were added to the eppendorf tube containing the total RNA (2.5 µg), incubated at 70°C for 10 minutes and immediately placed on ice for 2 minutes. The following were added: 4 µl of 5X First-strand buffer, 2 µl of 10mM dNTPs, 2 µl of 100 mM DTT and 1 µl of Power Script reverse transcriptase. After adding 100 µl sterile mineral oil, the tube was incubated at 42°C for 1 hour. The reaction was stopped by heating at 70°C for 15 minutes. The cDNA samples were aliquoted and stored in -80°C freezer.

3.2.4.5 RT-PCR analysis

RT-PCR analysis was done using the following reaction mixture: 15.4 µl of sterile Milli-Q water, 2.5 µl of 10X PCR buffer, 2.5 µl of 25mM $MgCl_2$, 0.5 µl of 10mM dNTPs, 0.5 µl of 10 uM forward primer, 0.5 ul of 10 µM reverse primer, 3 µl of cDNA and 0.1 µl of PFU polymerase or *Taq* polymerase. The sequences of primers used in RT-PCR analysis are shown in Table 3.1. The following RT-PCR profile was used: 1 cycle of 95°C for 2 minutes, 40 cycles of 95°C for 1 minute, 65°C for 1 minute and 72°C for 2 minutes and 1 cycle of 72°C for 5 minutes. The RT-PCR products were analyzed by 1.2 % agarose gel electrophoresis.

3.2.4.6 Cytokinin extraction, purification and HPLC/MS analysis

The cytokinins, extracted from the non-senescing, senescing and senesced leaves of the plants, were purified and analyzed using HPLC/MS following the method of Dobrev and Kaminek (2002) as previously described in Chapter 2.

3.3. Results

3.3.1 Biolistic transformation of rice

The compact, yellowish and globular embryogenic calli were formed from the scutellar tissue of the mature seeds within two weeks of inoculation on the callus induction media. After six weeks of callus induction (Figure 3.4 A), the embryogenic calli were selected and placed at the center of the Petri dish (Figure 3.4 B) for particle bombardment. The embryogenic calli were bombarded with pTRA132, a plasmid DNA containing the CAMV 35S-*hpt* gene, together with either pSG506, a plasmid DNA containing SAG12-*gusA* gene, or pSG516, a plasmid DNA containing SAG12-*ipt* gene. Plasmids pSG506 and pTRA132 were used in 45 sets of bombardments. The rice cultivar (and set of bombardment used for these experiments) were the following: IR64 (11), IR72 (10) and NPT lines, namely, IR66160-121-4-5-3 (4), IR65600-42-5-2 (4) and IR68552-100-1-2-2 (16). The last NPT line was fast-senescing during wet season 1999. Plasmid pSG516 was used in conjunction with pTRA132 for a total of 45 sets of bombardments. The chosen cultivars (and set of bombardments) used were the following: IR64 (8), IR72 (4), 3 NPT lines, namely, IR66160-121-4-5-3 (2), IR65600-42-5-2 (14) and IR 68552-100-1-2-2 (17). After one week, the bombarded calli were transferred to a selection mediumcontaining 50 µg/ml Hygromycin B antibiotic to kill the untransformed calli (Figure 3.4 C). The embryogenic calli which turned brown and died did not contain the *hpt* gene. However, the embryogenic calli which survived in the selection media may later give rise to putatively transformed plants. The surviving embryogenic calli were transferred to a plant regeneration medium (Figure 3.4 D) and plantlets were

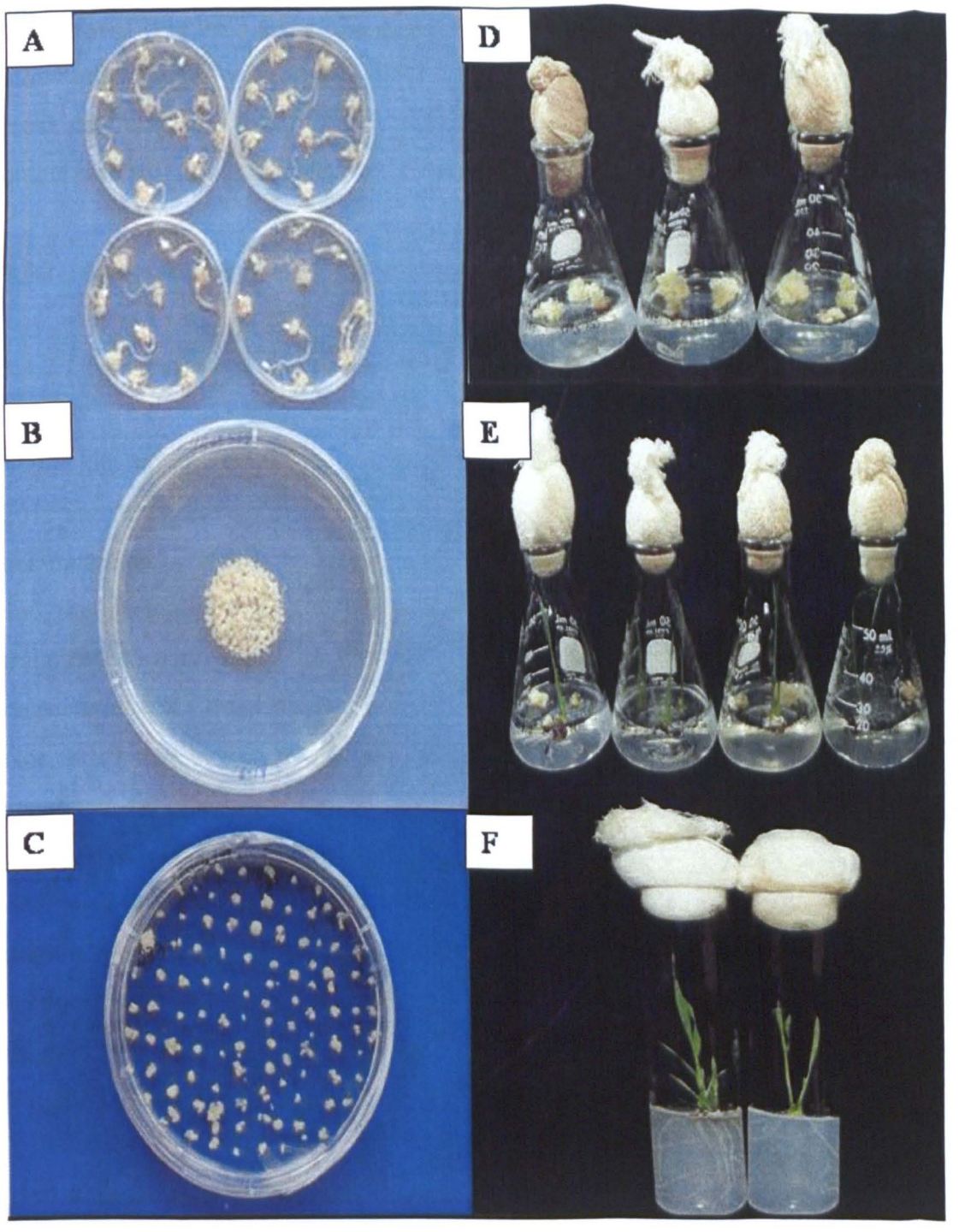

Figure 3.4 Different stages in the biolistic transformation of rice. (A) Six-week old calli growing on callus induction media; (B) The selected embryogenic calli were placed at the center of the Petri dish for particle bombardment; (C) The embryogenic calli bombarded with gold particles coated with the plasmid DNA constructs were transferred to a callus induction media containing 50mg/l hygromycin B; (D) The hygromycin-resistant calli were growing well on the callus induction media supplemented with 50mg/l hygromycin B; (E) Putative transgenic plantlets were growing on the regeneration media containing 50mg/l hygromycin B; (F) Putative transgenic plantlets produced roots when placed on the rooting media.

regenerated (Figure 3.4 E). The plantlets were transferred to a rooting media to allow more root formation (Figure 3.4 F). The plantlets were transferred in Yoshida's culture solution for 21 days. The plants were transferred in pots with autoclaved soil and grown until plant maturity.

3.3.2. Molecular analysis of the transgenic T_0 SAG12-*gusA* rice plants

3.3.2.1 Integration of the *gusA* gene in the transgenic T_0 rice plants

Stable integration of *gusA* and *hpt* genes into the genomes of transgenic plant Nos. 2019 and 2029, derived from IR 72, and plant No. 2027, derived from IR 64, was demonstrated by Southern blot analysis (Figure 3.5; Table 3.2). The plant DNA and the plasmid pSG506 digested with *NcoI* and *EcoRI* released a 1.87 kb fragment corresponding to the *gusA* gene (Figure 3.5 A, lanes 1-3 and 5-7, respectively). When probed with a *gusA* coding sequence, it was found that plant Nos. 2019 and 2029 had three copies of the *gusA* gene while plant No. 2027 contained only one copy of the *gusA* gene. Additional fragments indicated the presence of a rearranged copy in the rice genome. The *gusA* and *hpt* genes were integrated in plant Nos. 2019 and 2029. No bands were detected with the untransformed plant DNA (Figure 3.5A, lane 4). The membranes from this Southern blot were stripped and hybridised with an *hpt* probe (1.1 kb *BamHI* fragment of pTRA132). All the three transgenic plants contained the *hpt* gene (Figure 3.5B).

3.3.2.2 Expression of the *gusA* gene in the transgenic T_0 rice plants

RT-PCR analyses showed the presence of the *gusA* transcripts in the senesced, but not in the senescing flag leaf of plant No. 2027 (Figure 3.6B). However, there was also no GUS staining observed for the senesced leaf of plant No. 2027. Negative results were obtained for the senescing flag leaf and the senesced leaf of plant No. 2029 (Figure 3.6B). No staining was observed in panicles, leaf sheaths and

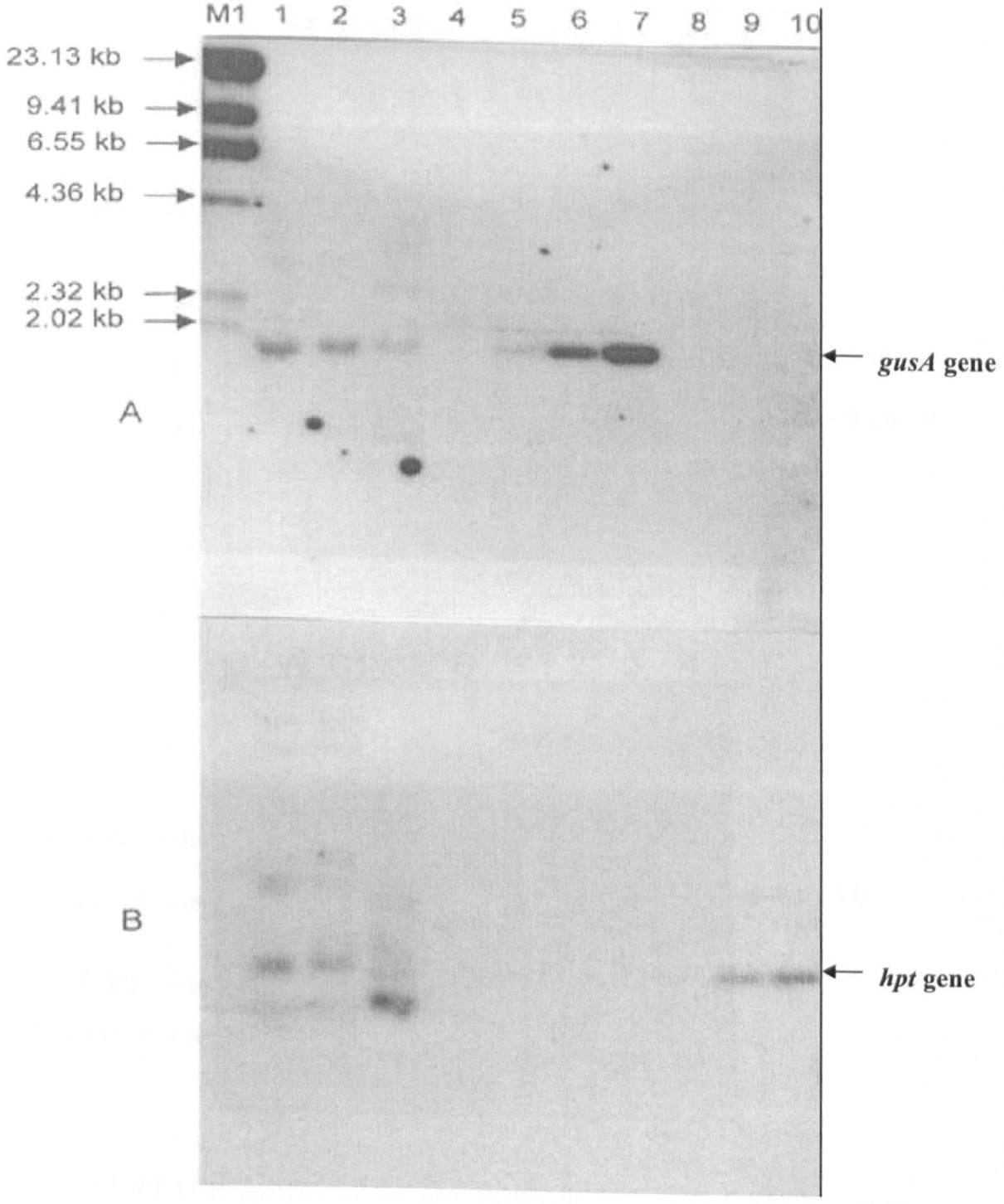

Figure 3.5 Southern blot analysis of T_0 rice plants transformed with *gusA* gene (A) and *hpt* gene (B). (M1) Lambda DNA cut with *Hind*III; Lanes 1 and 2 = plant Nos. 2019 and 2029 derived from IR72 rice variety; lane 3 = plant No. 2027 derived from IR64 rice variety, lane 4 = untransformed control plant; lanes 5 to 7 = 1, 5 and 10 copies of *gusA* gene, respectively; lanes 8 to 10 = 1, 5 and 10 copies of *hpt* gene, respectively.

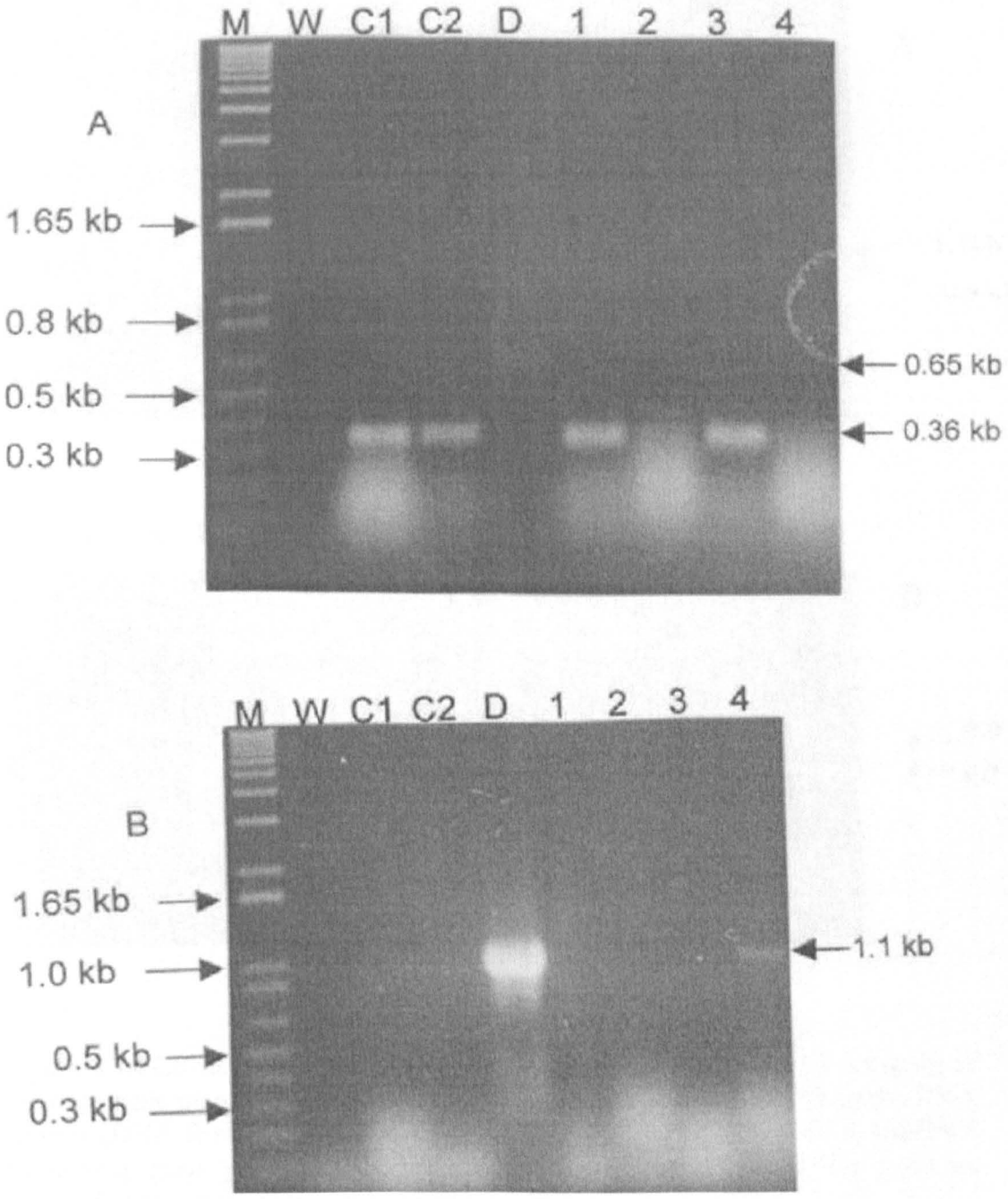

Figure 3.6 RT-PCR analysis of the glyceraldehyde-3-phosphate dehydrogenase (*g3pdh*) gene (A) and *gusA* gene (B) in the senescing and senesced leaves of transgenic rice plants transformed with SAG12-*gusA* gene. M = molecular weight marker (1 kb DNA ladder); W = water; C1 = senescing leaf from untransformed control plant; C2 = senesced leaf from untransformed control plant; D = DNA of the untransformed control plant; P = plasmid DNA containing the *gusA* gene; lane 1 = senescing leaf of plant No. 2029; lane 2 = senesced leaf of plant No. 2029; lane 3 = senescing leaf of plant No. 2027; lane 4 = senesced leaf of plant No. 2027.

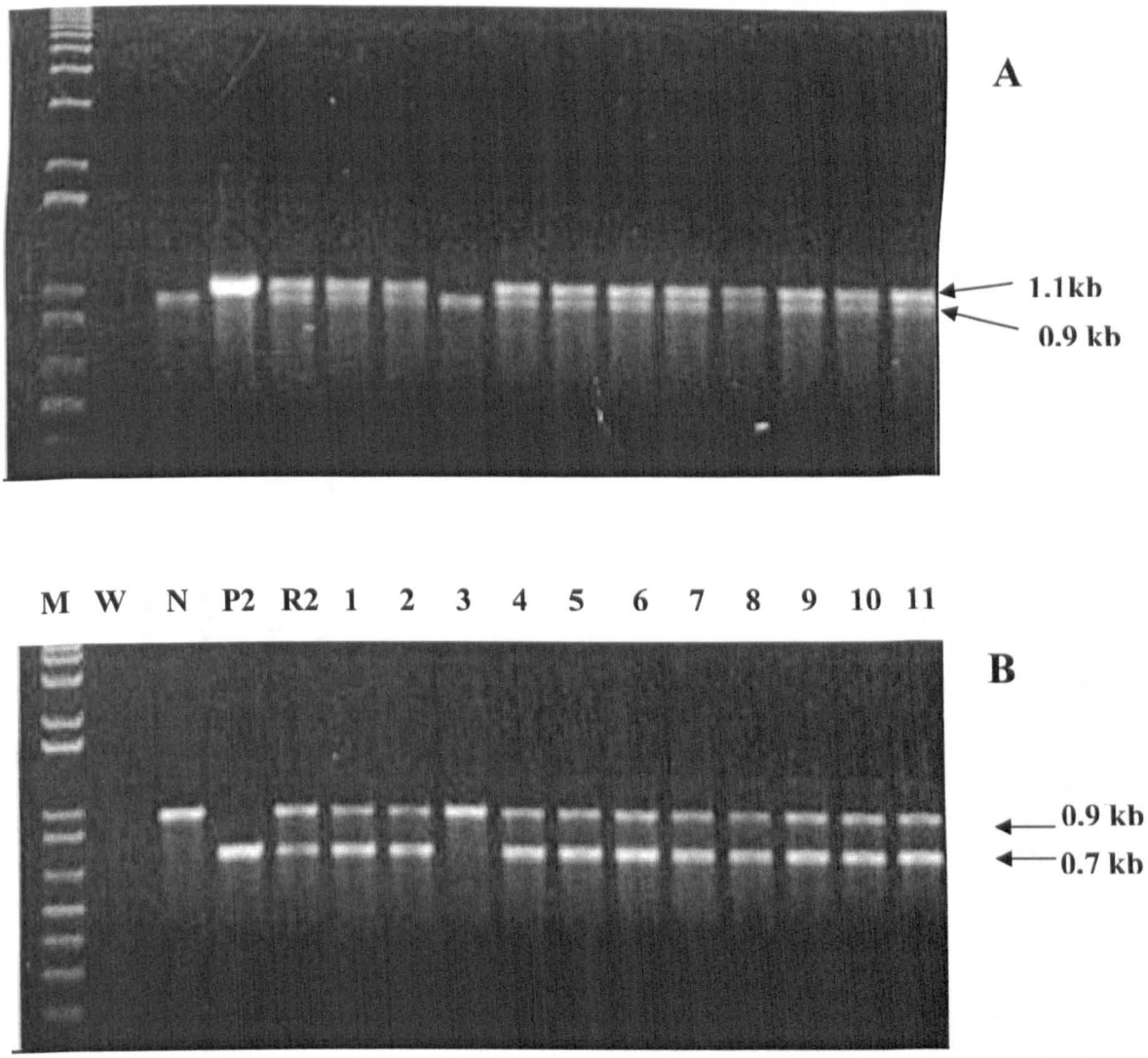

Figure 3.7 PCR analysis of the *gusA* gene (A) and *hpt* gene (B) in T_1 progeny of the transgenic plant No. T2029. W = no DNA control (water); N = negative DNA control (DNA from an untransformed rice plant); P1 = plasmid DNA (pSG506 containing *gusA* gene); R1 = reconstructed positive control (DNA from an untransformed rice plant and plasmid DNA containing *gusA* gene); control (water); N = negative DNA control (DNA from an untransformed rice plant); P2 = plasmid DNA (pTRA132 containing *hpt* gene); R2 = reconstructed positive control (DNA from an untransformed rice plant and plasmid DNA containing *hpt* gene); M = molecular size marker (1 kb DNA ladder); lanes 1 to 11 = T_1 progeny of plant No. 2029. The expected amplicon size was 0.9 kb for the presence of rice DNA and 1.1 kb for the presence of the *gusA* gene.

Table 3.2 Integration, expression and inheritance of the *gusA* gene in the transgenic rice plants.

Independent Transformant Number	**Experiment Number for Bombardment**	**Plant No.**	**Rice variety**	**PCR analysis of the *gusA* gene**	**Southern blot analysis of the *gusA* gene**	**RT-PCR analysis of the *gusA* gene**		**PCR analysis of the *gusA* gene in the T_1 progeny (*gusA*-positive:*gusA*-negative)**
						Senescing leaf	**Senesced leaf**	
1	179	2019	IR72	+	+	ND	ND	13:8
		2029	IR72	+	+	-	-	10:1
2	182	2027	IR64	+	+	-	+	sterile

ND=not determined

different types of leaves after the histochemical GUS assay which confirmed that the *gusA* gene was not expressed in plant No 2029 and No.2019 (data not shown). RT-PCR analysis of the *g3pdh* gene, encoding a glyceraldehyde-3-phosphate in rice, was done to check the purity of total RNA and the absence of any DNA. The expected size of the RT-PCR product of the *g3pdh* gene is 0. 35 kb using total RNA as template but it is 0.65 kb in the presence of any contaminating DNA in the total RNA. RT-PCR analysis showed the presence of the 0.35kb transcript in the RNA samples from plants which indicate the purity of total RNA used (Figure 3.6A).

3.3.2.3 Inheritance of the *gusA* gene in the transgenic T_1 rice plants

Inheritance of the introduced *gusA* and *hpt* genes from the two transgenic plants Nos. 2019 and 2029 into the T_1 generation was examined using PCR analysis. The *gusA* gene was inherited in 13 out of 21 T_1 progeny of plant No. 2019 (data not shown). The *gusA* and *hpt* genes were inherited in 10 out of 11 T_1 progeny of plant No. 2029 (Figure 3.7). This inheritance of the *gusA* gene does not follow the Mendelian segregation ratios which indicate that more T1 progeny should have been planted and analyzed. However, no expression was detectable with the histochemical GUS assay in various parts of the T_1 progeny containing the *gusA* gene (data not shown).

3.3.3. Molecular analysis of the transgenic T_0 SAG12-ipt rice plants

3.3.3.1 Integration of the *ipt* gene in the transgenic T_0 rice plants

PCR analysis showed the presence of 0.7 kb *ipt* gene in five T_0 plants (Figure 3.8). PCR analysis showed that the *ipt* gene was present in 13, 188, 4 and 5 clones for IR68552-100-1-2-2 from bombardment nos. 198-1, 198-3, 200-3 and 203-2, respectively (data not shown).

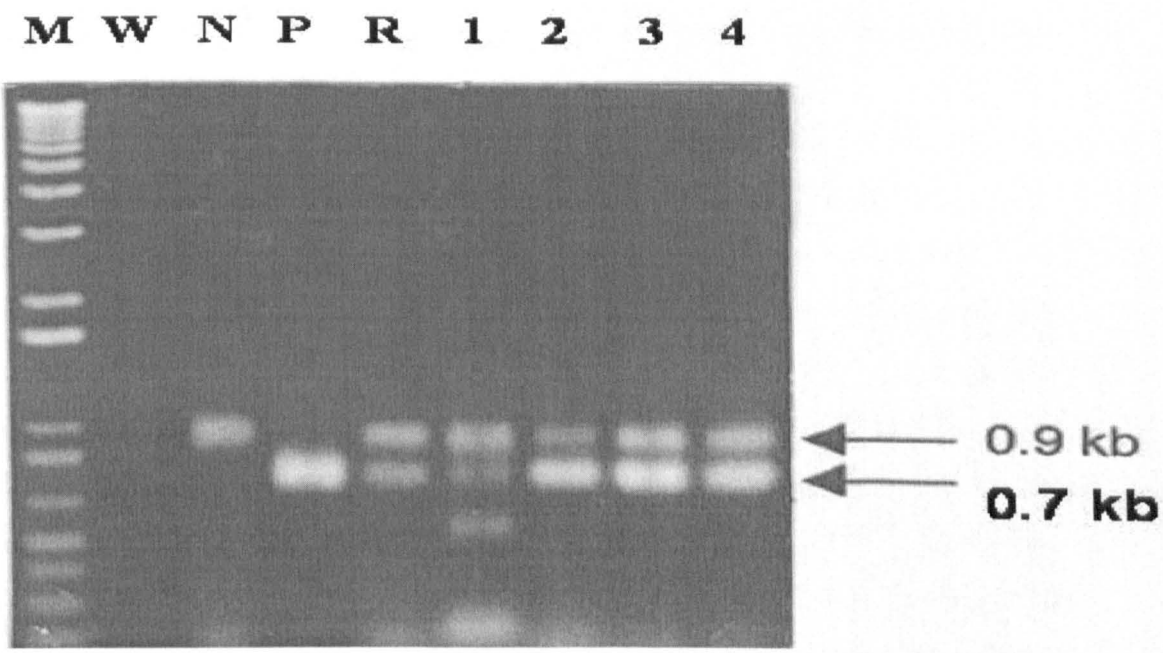

Figure 3.8 PCR analysis of the *ipt* gene in T_0 rice plants transformed with SAG12-*ipt* gene. W = no DNA control (water); N = negative DNA control (DNA from an untransformed rice plant); P = plasmid DNA (pSG516 containing SAG12-ipt gene); R=reconstructed positive control (DNA from an untransformed rice plant and plasmid DNA containing SAG12-*ipt* gene); M = 1 kb DNA ladder; lane 1 = plant No. T1147; lane 2 = plant No. T2013; lane 3 = plant No. T1194; lane 4 = plant No. T1193. The expected amplicon size was 0.9 kb for the presence of rice DNA and 0.7 kb for the presence of the *ipt* gene.

Southern blot analysis was done on a number of plants which were PCR-positive for the *ipt* gene. Southern blot analysis showed the presence of high molecular weight signal in the undigested genomic DNA of all the T_0 plants analysed except the control plant which indicate that the *ipt* gene was integrated into the genome of these T_0 plants (Figures 3.9, 3.10, 3.11 and 3.12). The genomic DNA of T_0 plants, control plant and plasmid pSG516, containing the *SAG12-ipt-nos* gene, were digested with *SpeI* which released a 3.18 kb fragment containing the *SAG12-ipt-nos* gene. The southern blots were probed with a 3.18 kb *SAG12-ipt-nos* probe. Southern blot analysis showed the integration of an intact copy of the *SAG12-ipt-nos* gene in the T_0 plants (Figures 3.9 to 3.12).

3.3.3.2 Expression of the ipt gene in the transgenic T_0 rice plants

The specificity of the *SAG12* promoter in the transgenic T_0 rice plants was determined by RT-PCR analysis and northern blot analysis in non-senescing unexpanded leaf, senescing fully expanded flag leaf at flowering and senesced fully expanded leaf. No result was obtained in Northern blot analysis of the transgenic plants, using 10, 20 and even 30ug of RNA, which may be due to low expression of the *ipt* gene, hence, RT-PCR analysis was used to analyzed the expression of the *ipt* gene. To ensure RNA was the real template, the extracted RNA was DNAse-treated to remove any DNA contamination. As a control, RT-PCR analysis was carried out using primers for the glyceraldehyde-3-phosphate dehydrogenase *(g3pdh)* gene which contains an intron. RT-PCR analysis showed 0.36 kb *g3pdh* amplicons (Figures 3.13A and 3.14) in the DNAse-treated RNA and 0.65 kb transcripts in the DNA (containing an intron), which indicate that the DNAse-treated RNA was not contaminated with DNA, hence, the RT-PCR analysis using *ipt* primers would give a reliable result.

RT-PCR analysis showed the presence of *ipt* transcripts in the leaves of the transformants (Figures 3.13B and 3.15; Table 3.3). The *ipt* gene was expressed in

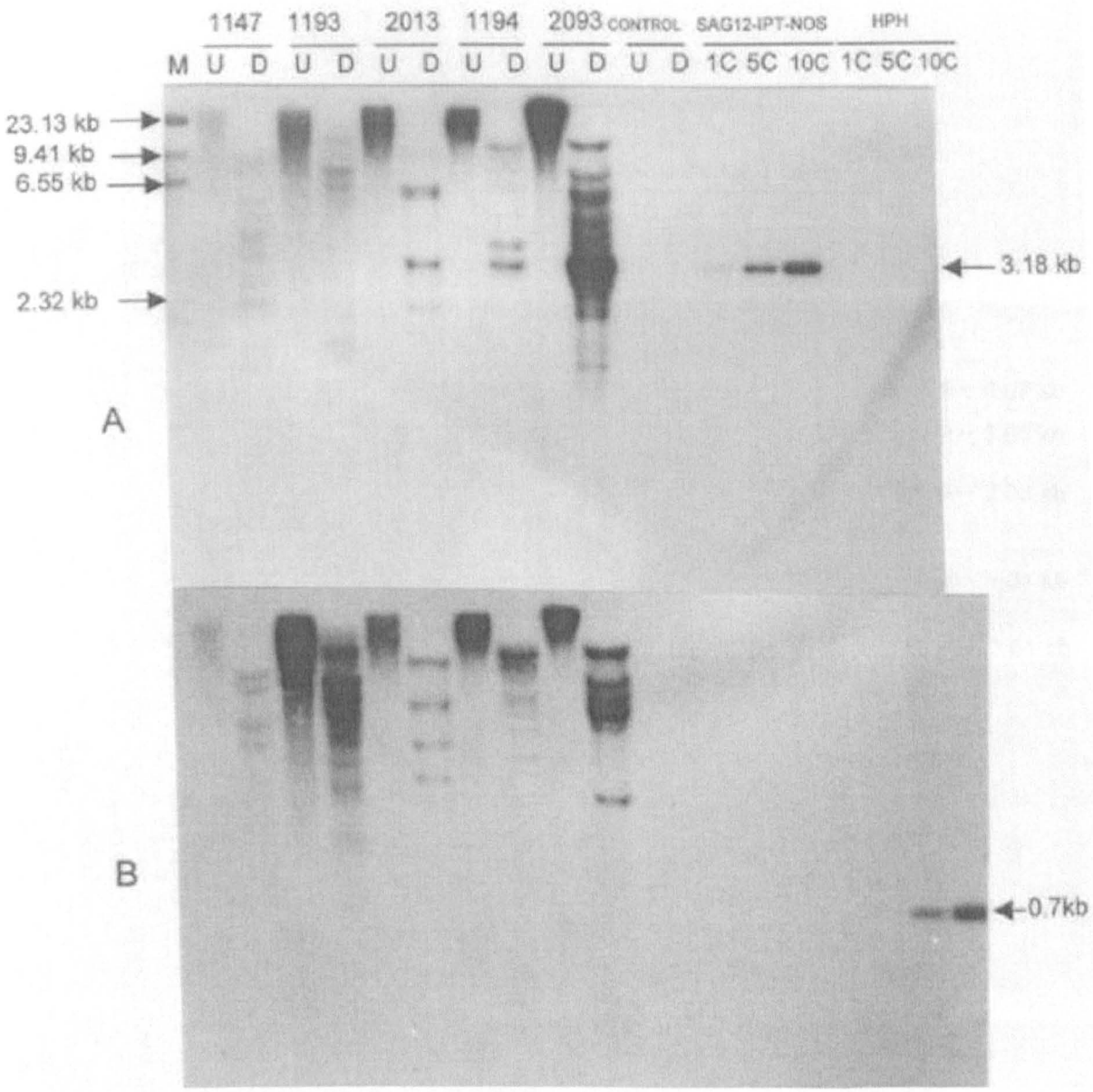

Figure 3.9 Southern blot analysis of transgenic T_0 rice plants transformed with *ipt* (A) and *hpt* (B) genes. M = Lambda DNA cut with *Hind*III; U = undigested plant DNA; D = digested plant DNA; Plant Nos. T1147, T1193 and T2013, respectively, derived from NPT line IR65600-42-5-2; plant No. T1194 derived from NPT line IR66160-121-4-5-3; plant No. T2093 derived from NPT line IR68552-100-1-2-2; C = copy or copies of 3.18 kb *SAG12-ipt-nos* gene or 0.7 kb *hph* gene, respectively.

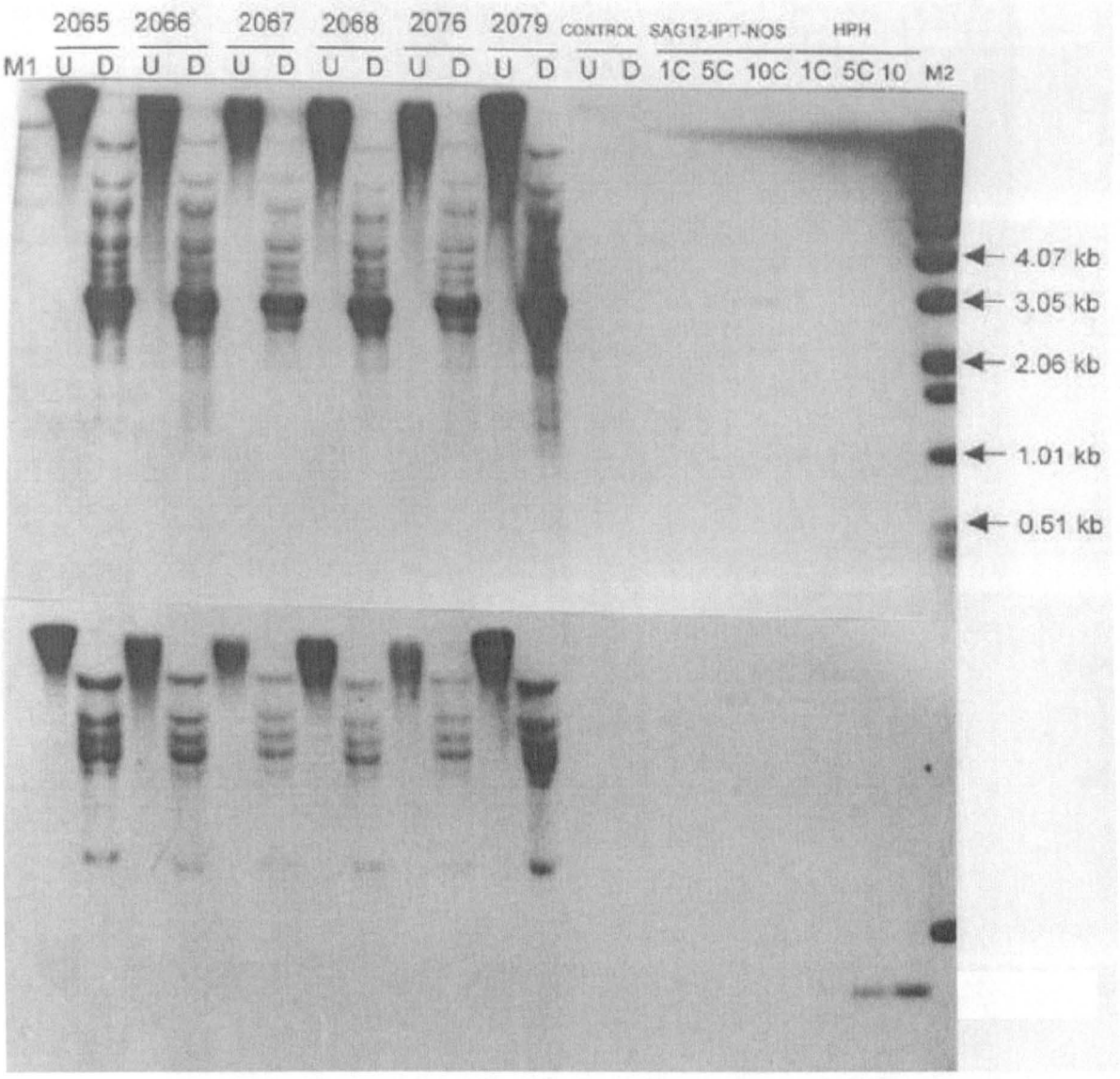

Figure 3.10 Southern blot analysis of transgenic rice plant Nos. 2065, 2066, 2067, 2068 and 2079, derived from NPT line IR68552-100-1-2-2, transformed with *SAG12-ipt-nos* (A) and *hph* (B) genes.

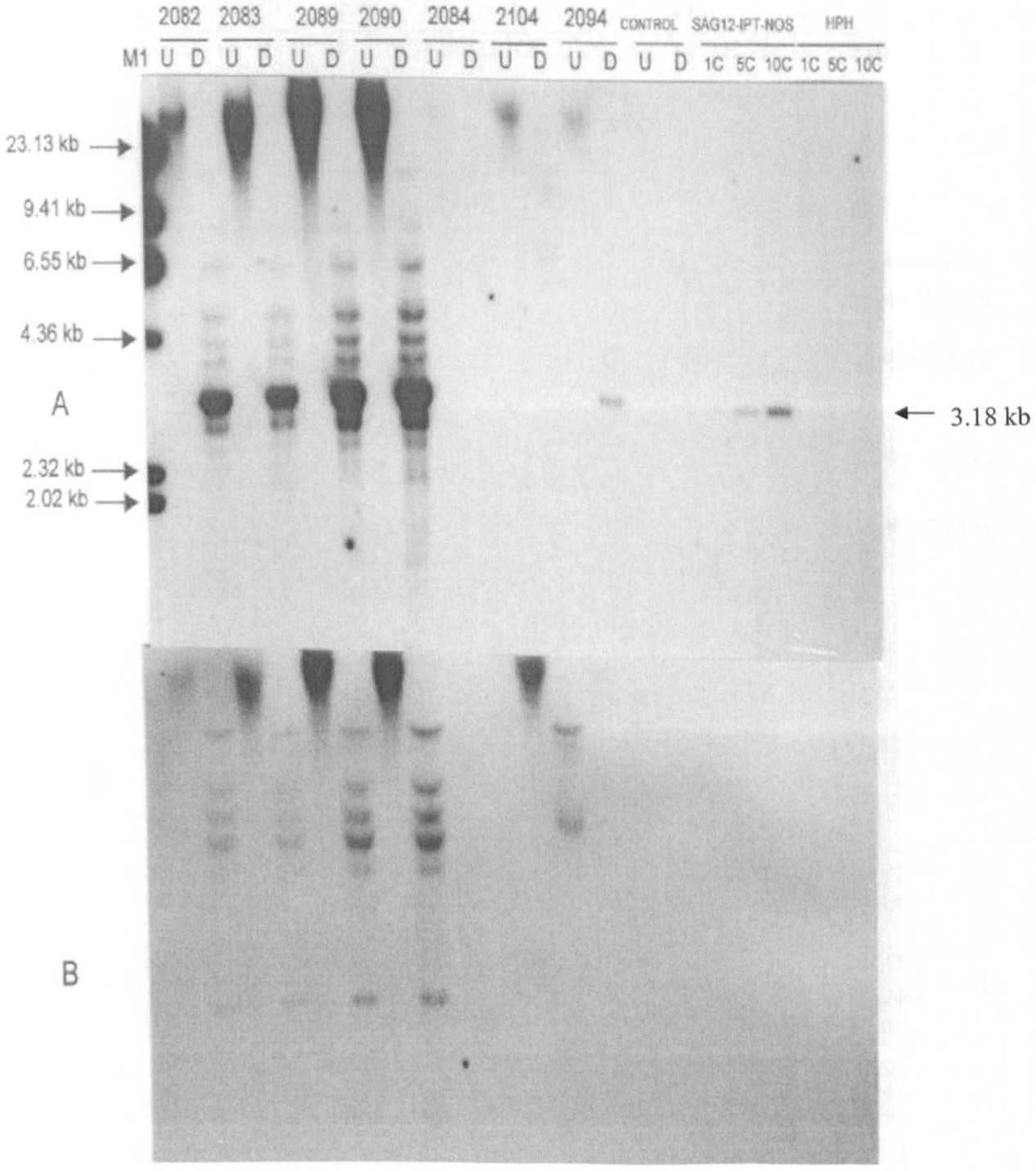

Figure 3.11 Southern blot analysis of transgenic rice plant Nos. 2082, 2083, 2089, 2104 and 2094, derived from NPT line IR68552-100-1-2-2, transformed with *SAG12-ipt-nos* (A) and *hph* (B) genes.

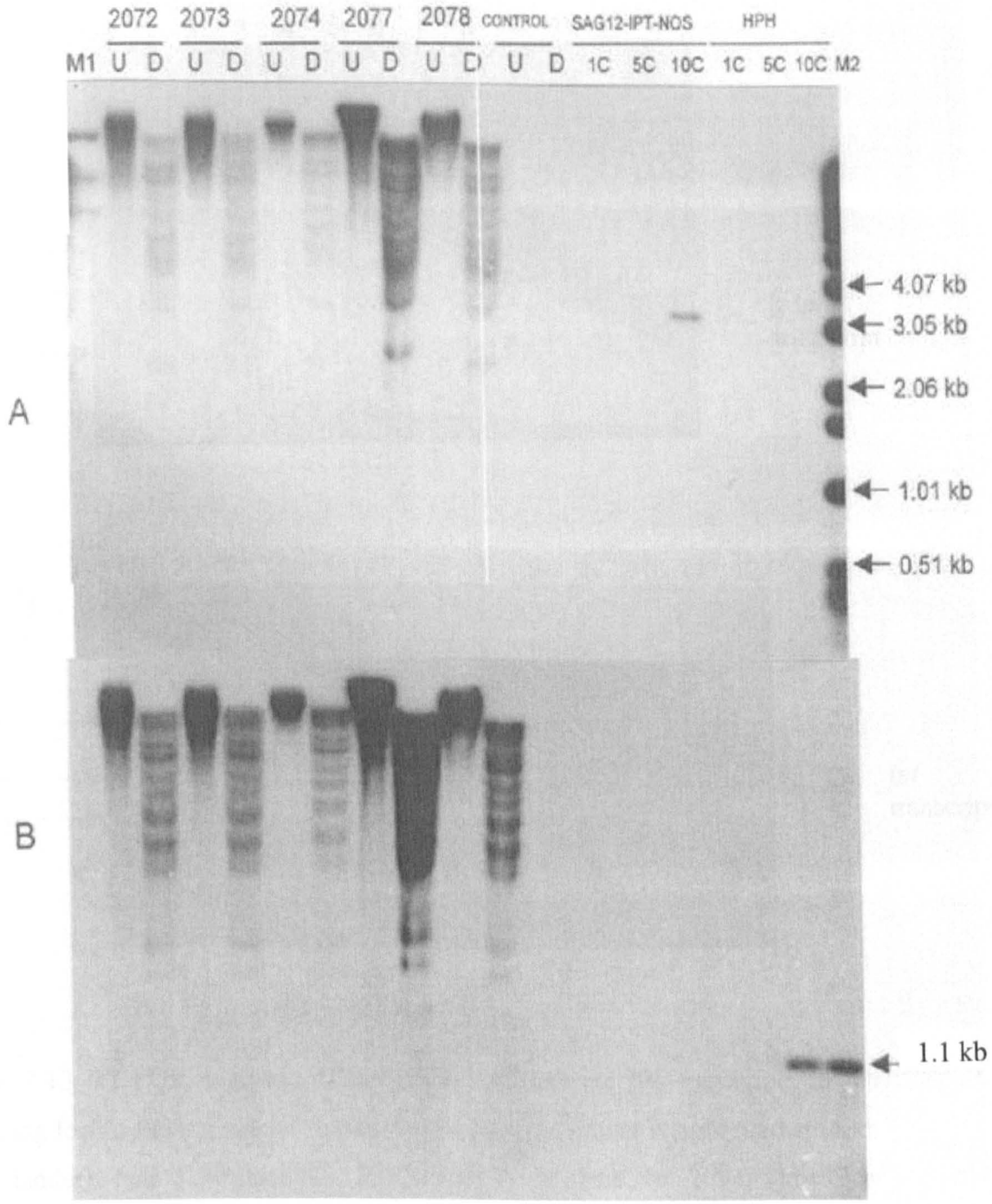

Figure 3.12 Southern blot analysis of transgenic rice plant Nos. 2072, 2073, 2074, and 2078, derived from NPT line IR68552-100-1-2-2, transformed with *SAG12-ipt-nos* (A) and *hph* (B) genes.

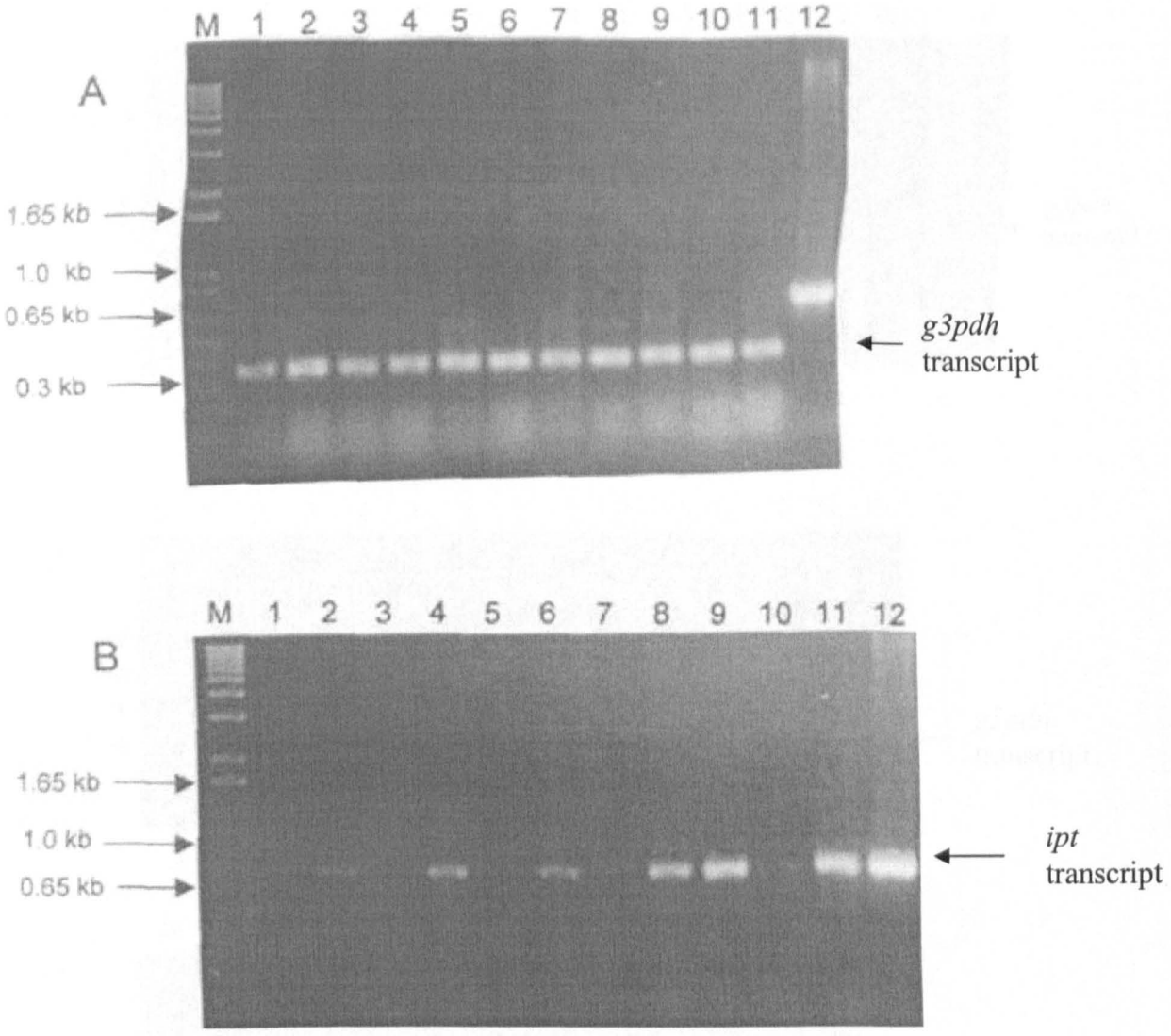

Figure 3.13 RT-PCR analysis of the *g3pdh* (A) and *ipt* (B) transcripts in the senescing leaf of the transgenic T_0 rice plants. M = molecular weight marker (1 kb DNA ladder); lane 1 = plant No. 2072; lane 2 = plant No. 2094; lane 3 = untransformed control plant; lane 4 = plant No. 2104; lane 5 = plant No. 2013; lane 6 = plant No. 1193; lane 7 = plant No. 2133; lane 8 = plant No. 1147; lane 9 = plant No. 1194; lane 10 = plant No. 2077; lane 11 = plant No. 2521; lane 12 = DNA of plant No. 1193.

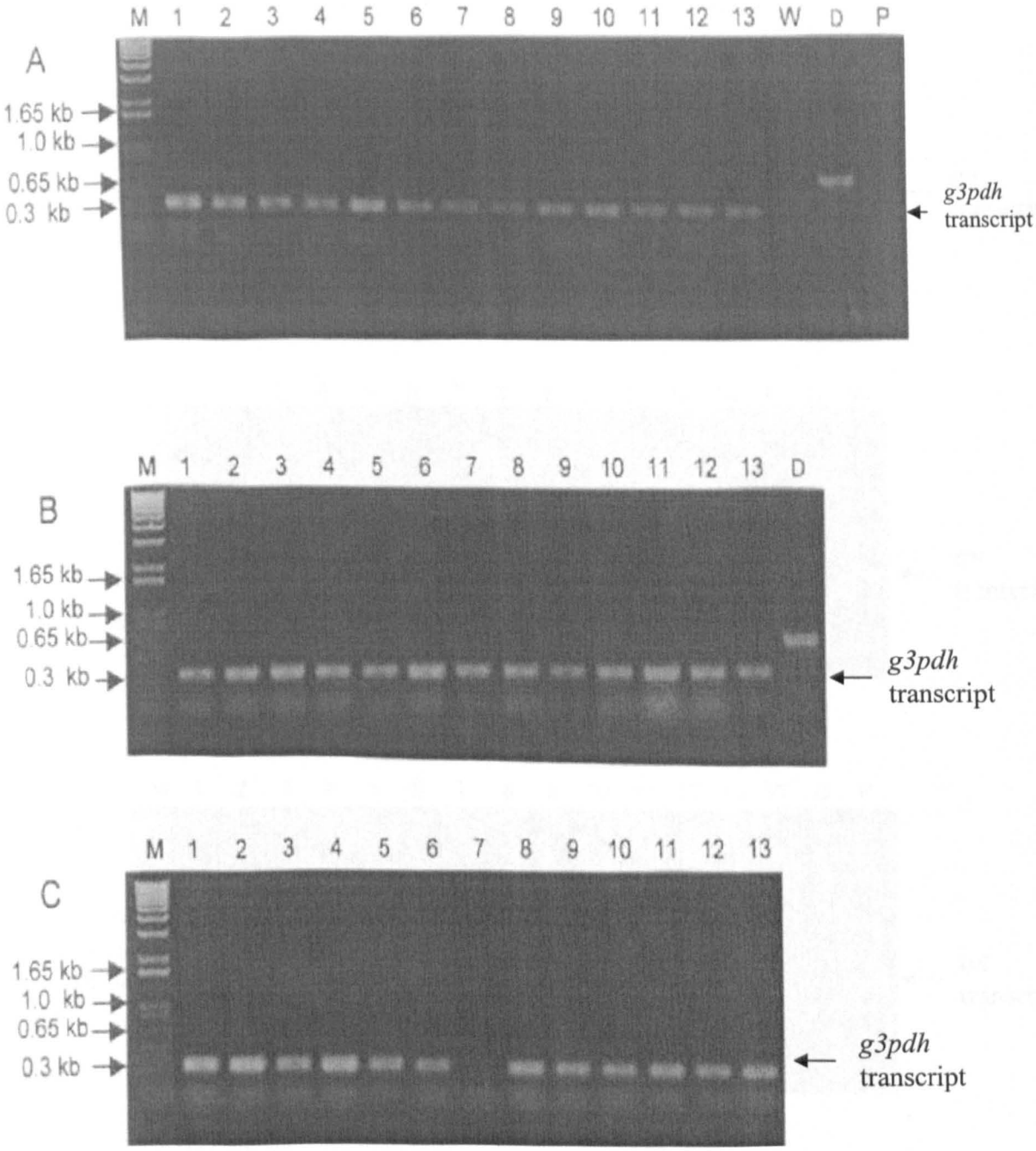

Figure 3.14 RT-PCR analysis of the *g3pdh* transcripts in the non-senescing (A), senescing (B) and senesced (C) leaves of the transgenic T_0 rice plants. M = molecular weight marker (1 kb DNA ladder); lane 1 = plant No. 1147; lane 2 = plant No. 1193; lane 3 = plant No. 1194; lane 4 = plant No. 2013; lane 5 = plant No. 2104; lane 6 = plant No.2094; lane 7 = plant No. 2072; lane 8 = control plant; lane 9 = plant No. 2076; lane 10 = plant No. 2093; lane 11 = plant No. 2068; lane 12 = plant No. 2089; lane 13 = plant No. 2078; W = water; D = DNA of control plant; P = pSG516 containing the *ipt* gene.

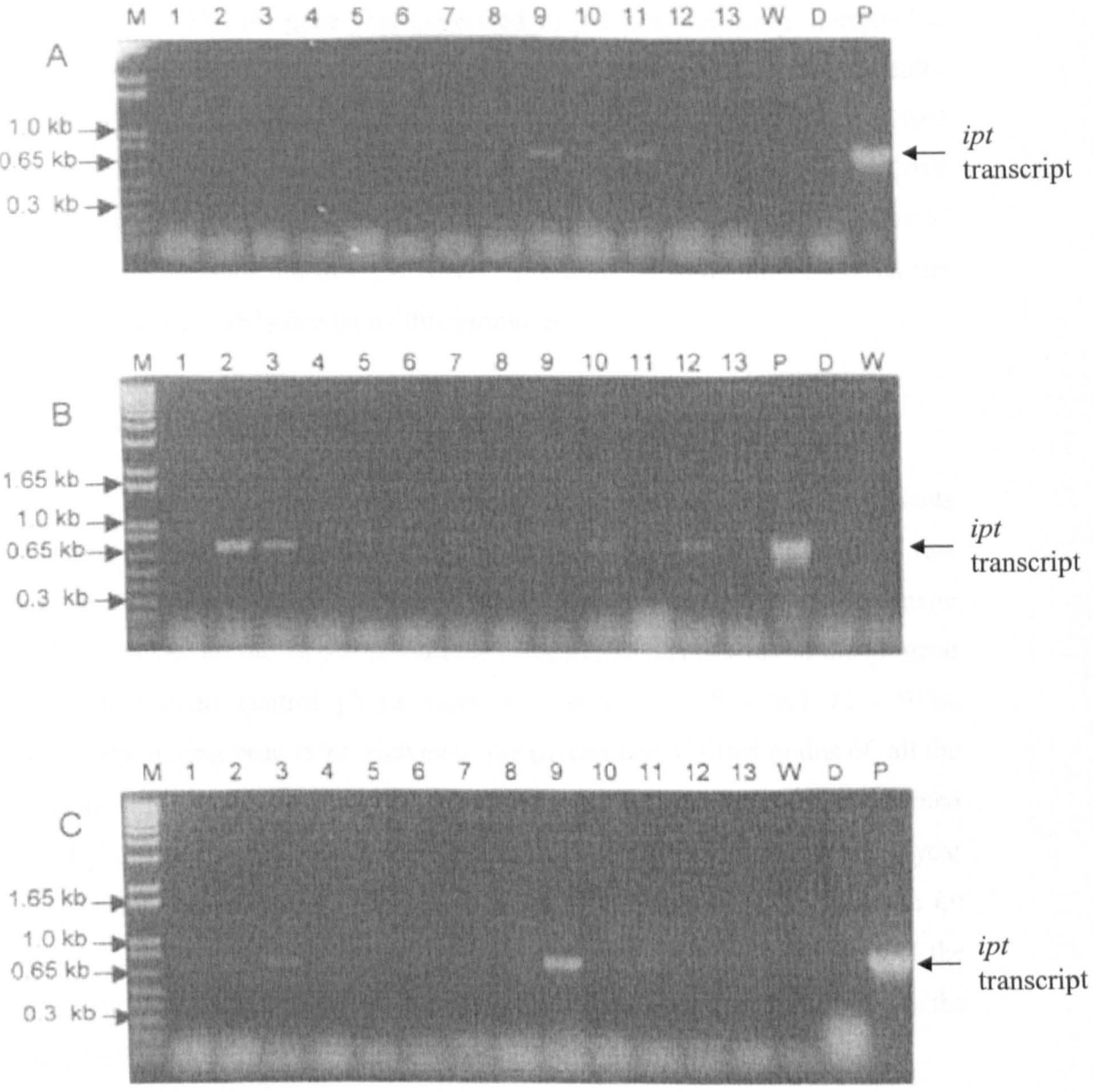

Figure 3.15 RT-PCR analysis of the *ipt* transcripts in the non-senescing (A), senescing (B) and senesced (C) leaves of the transgenic T_0 rice plants. M = molecular weight marker (1 kb DNA ladder); lane 1 = plant No. 1147; lane 2 = plant No. 1193; lane 3 = plant No. 2013; lane 4 = plant No. 2133; lane 5 = plant No. 2104; lane 6 = plant No.2094; lane 7 = plant No. 2072; lane 8 = control plant; lane 9 = plant No. 2076; lane 10 = plant No. 2094; lane 11 = plant No. 2068; lane 12 = plant No. 1194; lane 13 = plant No. 2078; W = water; D = DNA of control plant; P = pSG516 containing the *ipt* gene.

the senescing expanded leaves of plant Nos. 2133, 2094, 1193, 1147 and 1194 (Figures 3.13B). The *ipt* gene was expressed in the non-senescing unexpanded leaves of plant Nos. T2076, T2093, T2068 and T2089 plants, senescing fully expanded leaves of flowering plant Nos. T1193, T1194, T2093, T2068 and T2089 plants and in senesced leaves of plant Nos. T1194, T2076, T2068 and T2089 plants (Figure 3.15). The expression of the *ipt* gene in non-senescing leaves of four transformants indicate that the SAG12 promoter is sometimes leaky so the *ipt* gene was not properly driven by this promoter.

3.3.3.3 Grain filling percentage in the transgenic T_0 rice plants

Table 3.3 shows that the grain filling percentage of the transgenic T_0 rice plants was lower than the control plants. But a high percentage of the transgenic T_0 rice plants were completely sterile or highly sterile (data not shown). The percentage of filled grains of all the T_0 plants with the *ipt* gene, T_0 plants without the *ipt* gene and untransformed control plants were 2 - 56%, 2 - 78% and 73 - 91%. respectively, during year 1999. However, the percentage of filled grains of all the T_0 plants with the *ipt* gene, T_0 plants without the *ipt* gene and untransformed control plants were and 0 - 44%, 0 -74% and 74 - 94%, respectively, during year 2000.The high sterility and wide range of fertility in the T_0 plants with the *ipt* gene and T_0 plants without the *ipt* gene indicate that sterility is an effect of the tissue culture and the wide range of fertility indicates that it is due to the somaclonal variation among these plants regenerated from tissue-culture.

3.3.3.4 Cytokinin levels in the transgenic T_0 rice plants

Cytokinins (CKs) in the non-senescing, senescing and senesced leaves of primary transformants, namely, T2068 and T1193 plants were analysed. Twenty two forms of CKs, identified in the three leaves, were placed into four different groups of CKs, namely, (1) active CKs, (2) inactive CKs, (3) *cis* derivatives of zeatin and (4) storage CKs. The active CKs observed were Z, DHZ, DHZR, iP and iPR. The

Table 3.3. Integration, expression and inheritance of the *ipt* gene in transgenic SAG12-*ipt* T_0 plants.

Plant number	**Southern blot analysis of the *ipt* gene in the T_0 plants**	**RT-PCR analysis of the *ipt* gene in the T_0 plants**			**Percentage of filled grain (%)**	**Weight of 1000 grains (g)**	**PCR analysis of the *ipt* gene in the T_1 plants (*ipt*-positive:*ipt*-negative)**
		Non-senescing leaf	**Senescing leaf**	**Senesced leaf**			
1147[1]	+	-	+	-	33.82	26.67	17:10
1193[1]	+	-	+	-	43.52	26.49	27:9
2013[1]	+	-	+	+	0.88	Sterile	sterile
Control[1]	-	-	-	-	75.20	25.73	-
1194[2]	+	+	+	+	2.45	17.50	sterile
Control[2]	-	-	-	-	65.71	25.14	-
2104[3]	+	-	+	-	22.50	19.44	20.2
2094[3]	+	+	+	-	0.00	0.00	-
2072[3]	+	-	+	-	ND	ND	sterile
2068[3]	+	+	+	+	17.45	26.63	17:11
Control[3]	-	-	-	-	76.96	32.20	-

[1]IR65600-42-5-2; [2]IR66160-121-4-5-3; [3]IR68552-100-1-2-2; ND=not determined

inactive CKs observed were Z7G, DHZ7G, Z9G, DHZ9G, iP7G and iP9G. The *cis* derivatives of zeatin observed were *c*-Z7G, *c*-Z9G, *c*-ZOG, *c*-ZROG, *c*-Z and *c*-ZR. The storage CKs observed were ZOG, Z9ROG, DHZROG and DHZOG.

The levels of the four groups of CKs vary in the non-senescing, senescing and senesced leaves of plant Nos. T2068 and T1193. The active CKs in the non-senescing leaf and storage CKs in the senesced leaf were much higher in the transgenic plant No.2068 compared to the control plant (Figure 3.16). The levels of active and storage CKs in the senesced leaves were higher in plant No.1193 compared to the control plant (Figure 3.17). the contents of the four major groups of CKs in the non-senescing and senesced leaves were lower in transgenic plant No. T1193 compared to the control plants.

3.3.3.4 Inheritance of the *ipt* gene in T_1 progeny of transgenic rice plants

The inheritance of the *ipt* gene in the T_1 progeny of transgenic rice plants is shown in Table 3.3. The *ipt* gene was inherited in a 3:1 ratio in plant Nos. 1193 (Figure 3.19) which indicate that the *ipt* gene is inherited in a Mendelian ratio. The *ipt* gene was inherited in a 16:10 ratio in plant No. T1147 (Figure 3.18), in a 17:10 ratio in plant No. T2068 and in a 11:1 ratio in plant No. 2104. However, the *ipt* gene was not inherited in plant Nos. 2013, 1194 and 2072which may indicate that the plants are sterile.

3.3.3.5 Phenotype and inheritance of the *ipt* gene in T_1 and T_2 progeny of transgenic plant No. T1193

PCR analysis showed that the *ipt* gene was inherited in a 3:1 segregation ratio in the T_1 progeny of plant No. T1193 which indicates that the *ipt* gene was inherited as a single Mendelian locus (Figure 3.19). Among the 27 *ipt*-positive T_1 plants, 23 of them displayed morphology and development which are the same like the nine *ipt*-negative T_1 plants and control plants. However, four *ipt*-positive T_1 plants

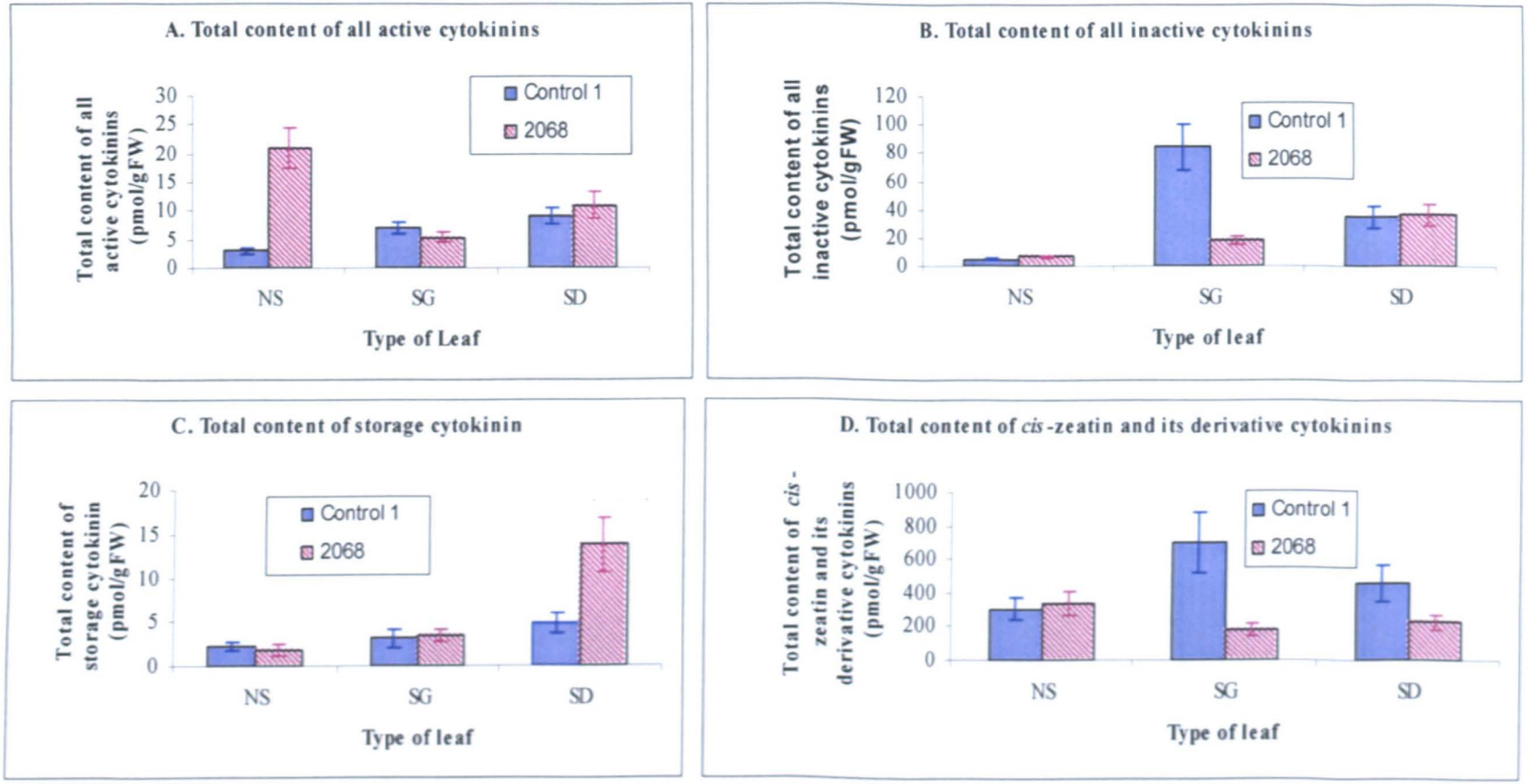

Figure 3.16. The total content of active (A), inactive (B), storage (C) and *cis*-zeatin and its derivative cytokinins (D) in the non-senescing (NS), senescing (SG) and senesced (SD) leaves of transgenic T2068 plant (striped bar) and control 1 plant (non-striped bar).Total content of active cytokinins = Z, DHZ, Z9R, DHZ9R, IP and IP9R; Total content of inactive cytokinins = Z7G,Z9G, DHZ7G, DHZ9G, IP7G and IP9G; Total content of storage cytokinins = DHZOG, ZOG, Z9ROG and DHZ9ROG; Total content of *cis*-zeatin and its derivatives = *c*-Z, *c*-Z7G, *c*-Z9G, *c*-ZOG, *c*-ZROG and *c*-Z9R.

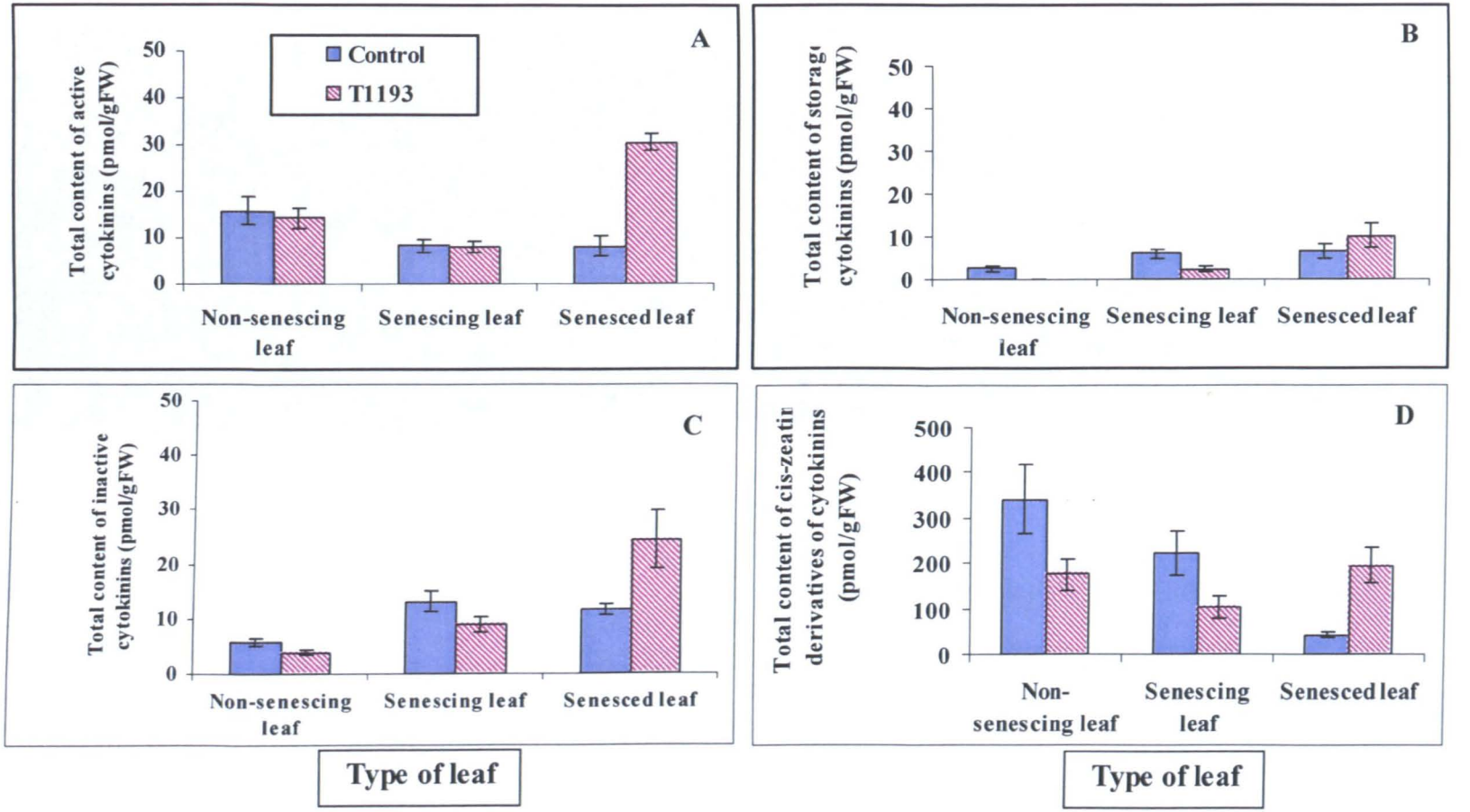

Figure 3.17. The total content of active (A), inactive (B), storage (C) and *cis*-zeatin and its derivative cytokinins (D) in the non-senescing (NS), senescing (SG) and senesced (SD) leaves of transgenic T1193 plant (striped bar) and control 1 plant (non-striped bar). Total content of active cytokinins = Z, DHZ, Z9R, DHZ9R, iP and iP9R; Total content of storage cytokinins = DHZOG, ZOG, Z9ROG and DHZ9ROG; Total content of inactive cytokinins = Z7G,Z9G, DHZ7G, DHZ9G, iP7G and iP9G; Total content of *cis*-zeatin and its derivatives = *c*-Z, *c*-Z7G, *c*-Z9G, *c*-ZOG, *c*-ZROG and *c*-Z9R.

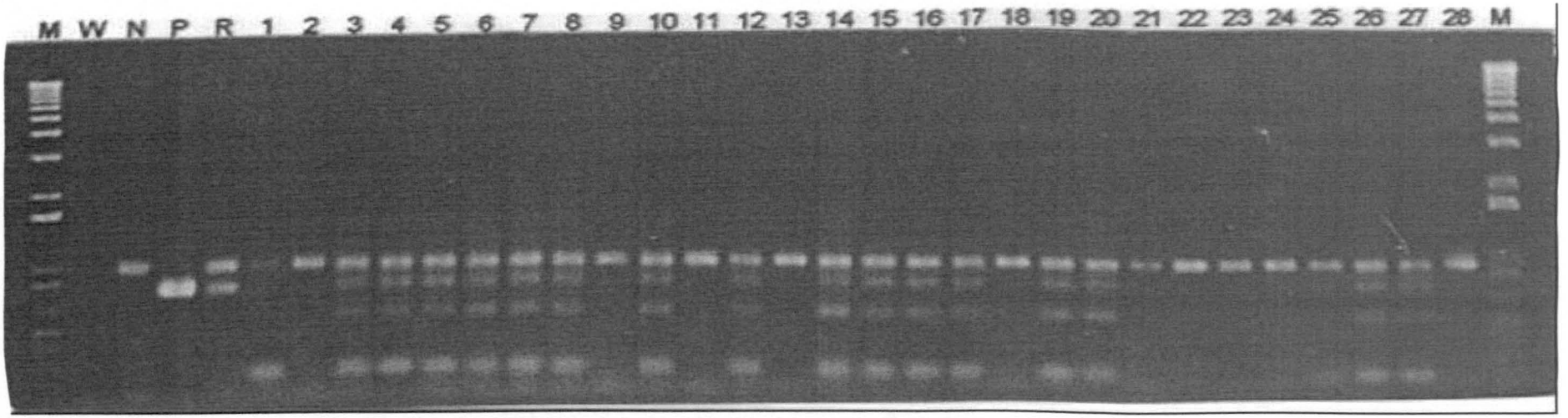

Figure 3.18 PCR analysis of the *ipt* gene in T_1 progeny of transgenic line T1147. W = no DNA control (water); N = negative DNA control (DNA from an untransformed rice plant); P = plasmid DNA (pSG516 containing SAG12-*ipt* gene); R = reconstructed positive control (DNA from an untransformed rice plant and plasmid DNA containing the *ipt* gene); M = molecular size marker (1 kb DNA ladder); lanes 1 to 28 = T_1 progeny of transgenic line T1147. Using RG100 primers, the expected amplicon size was 0.9 kb for the presence of rice DNA in the untransformed control rice plant (lane N) and all the T_1 plants (lanes 1-26). Using *ipt* primers, the expected amplicon size was 0.7 kb for the presence of the *ipt* gene in the plasmid pSG516 positive control, (labelled P), and in T_1 plants which inherited the *ipt* gene.

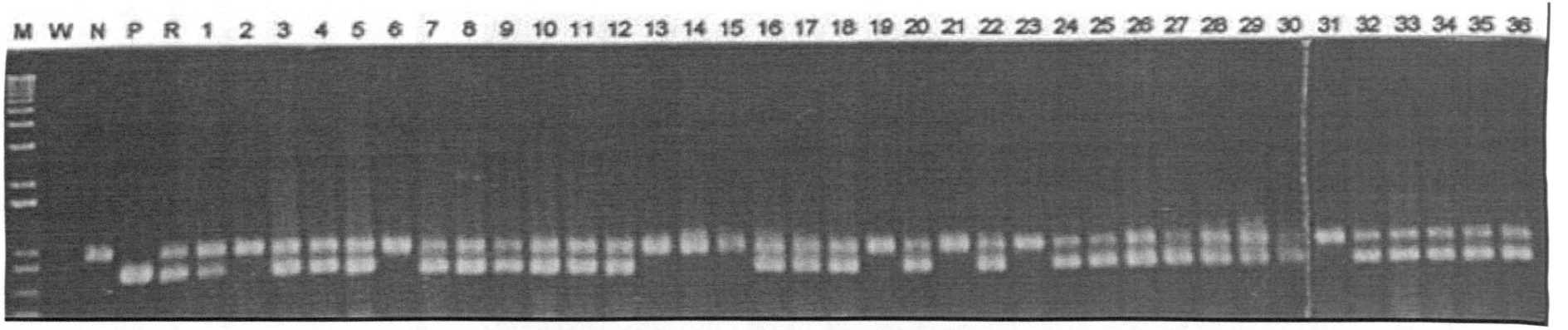

Figure 3.19 PCR analysis of the *ipt* gene in T_1 progeny of transgenic line T1193. W = no DNA control (water); N = negative DNA control (DNA from an untransformed rice plant); P = plasmid DNA (pSG516 containing SAG12-ipt gene); R = reconstructed positive control (DNA from an untransformed rice plant and plasmid DNA containing the *ipt* gene); M = molecular size marker (1 kb DNA ladder); lanes 1 to 36 = T_1 progeny of transgenic line T1193. Using RG100 primers, the expected amplicon size was 0.9 kb for the presence of rice DNA in the untransformed control rice plant (lane N) and all the T_1 plants (lanes 1-26). Using *ipt* primers, the expected amplicon size was 0.7 kb for the presence of the *ipt* gene in the plasmid pSG516 positive control, (labelled P), and in T_1 plants which inherited the *ipt* gene.

Figure 3.20 The phenotype of the T_1 progeny of transgenic line T1193. (A) The 36 T_1 progeny of transgenic line T1193. (B) The representative phenotype of the untransformed control plant (plant number 1), T_1 progeny of line T1193 which does not have the *ipt* gene (plant number 2), T_1 progeny of line T1193 which contain the *ipt* gene (plant number 3). T_1 progeny of line T1193 which contain the *ipt* gene (plant number 4) and showed shorter plant height, delayed flowering time and delayed leaf senescence.

showed altered phenotype (Figure 3.20). The four *ipt*-positive T_1 plants, namely, line Nos. 1193-7, 1193-24, 1193-27 and 1193-36, showed delayed flowering, (82%), followed by nine *ipt*-negative T_1 plants (55%), and 23 *ipt*-positive T_1 plants (49%). However, four *ipt*-positive T_1 plants with delayed flowering and even delayed leaf senescence at maturity gave poor fertility (3%). PCR analysis was wrinkled leaf, delayed leaf senescence and poor spikelet fertility. The percentage of filled grains was highest for the control plants done in T_2 progeny of these four lines. Two of these lines, line Nos. 1193-36 and 1193-24 were homozygous-positive for the *ipt* gene, while the other two lines, line Nos. 1193-27 and 1193-7 were heterozygous-positive for the *ipt* gene. Delayed flowering, delayed leafsenescence and poor spikelet fertility were also observed in the T_2 progeny of these lines. These results indicate that the presence of the *ipt* gene did not enhance the grain filling percentage of the plants. The delayed leaf senescence, delayed flowering and poor grain filling percentage in the four T_1plants and their T_2 progeny indicate that there might be overexpression of the *ipt* gene in these plants.

3.3.6 Cytokinin levels in the leaves of the T_2 progeny of plant No. T1193

Cytokinins (CKs) in the pre-senescing, senescing and senesced leaves of two T_2 progeny of T1193, namely, T1193-24-9 and T1193-27-8 plants were analysed. Cytokinin analysis of the T_2 progeny of T1193 plant showed that the levels of Z9R and IP in the leaves of T1193-24-9 plant and the levels of Z9R, DHZ9R, iP and iP9R in the leaves of T1193-27-8 plant were higher than the control plant. The levels of inactive CKs, storage CKs and *cis* derivatives of zeatin in the senesced leaves of T1193-24-9 plant were 7.08-fold, 6.10-fold and 6.87-fold higher, respectively, than in control plant (Figure 3.21). The levels of the four groups of CKs in the non-senescing and senescing leaves were similar in T1193-24-9 and control plants were similar. The levels of inactive CKs in the pre-senescing, senescing and senesced leaves of T1193-24-9 plant were 1.60-fold, 2.34-fold and 7.08-fold higher, respectively, than in control plant (Figure 3.21). The levels of storage and *cis*-zeatin derivatives of zeatin were 6.10-fold and 6.87-

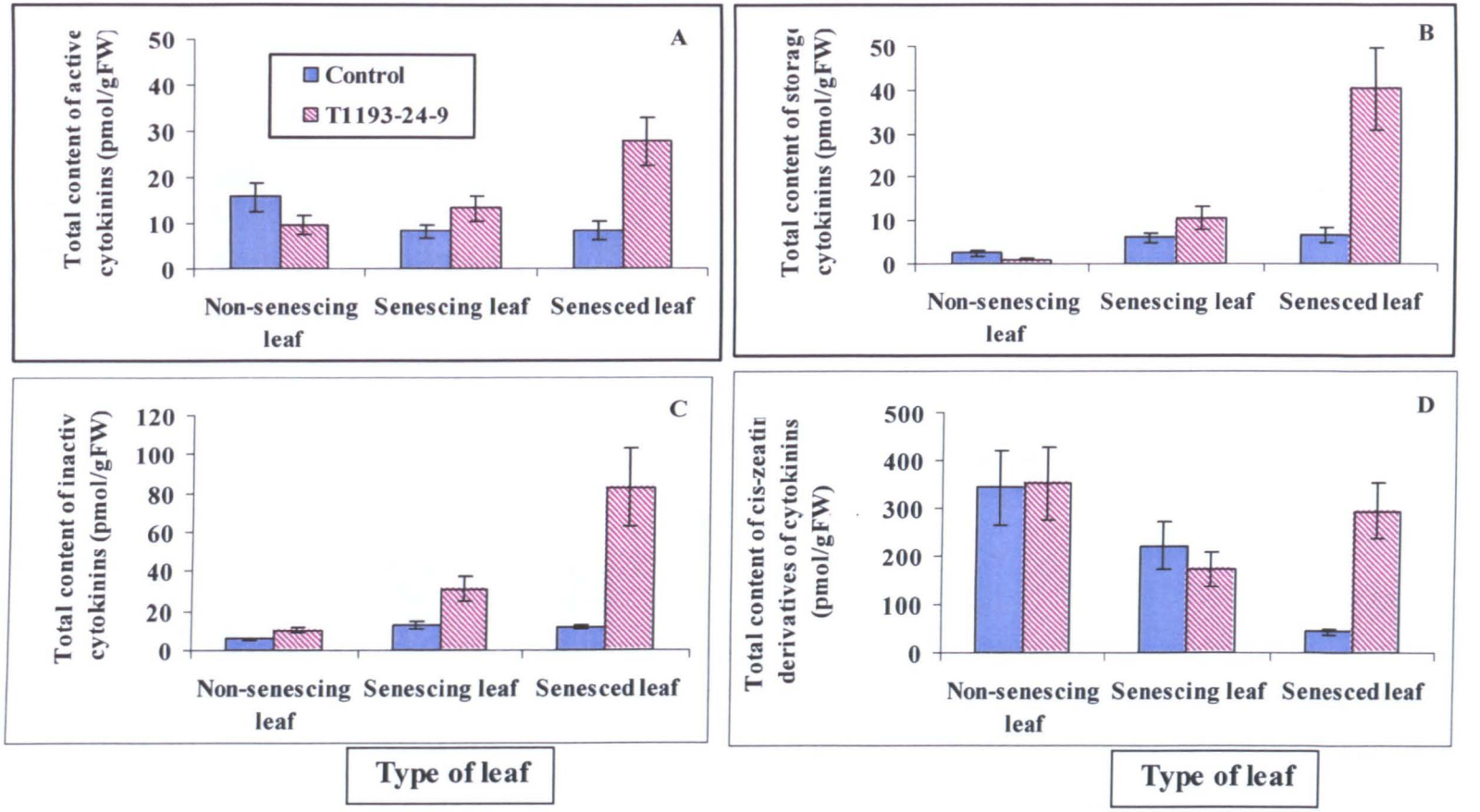

Figure 3.21. The total content of active (A), inactive (B), storage (C) and *cis*-zeatin and its derivative cytokinins (D) in the non-senescing (NS), senescing (SG) and senesced (SD) leaves of transgenic T1193-24-9 plant (striped bar) and control 1 plant non-striped bar). Total content of active cytokinins = Z, DHZ, Z9R, DHZ9R, iP and iP9R; Total content of storage cytokinins = DHZOG, ZOG, Z9ROG and DHZ9ROG; Total content of inactive cytokinins = Z7G,Z9G, DHZ7G, DHZ9G, iP7G and iP9G; Total content of *cis*-zeatin and its derivatives = *c*-Z, *c*-Z7G, *c*-Z9G, *c*-ZOG, *c*-ZROG and *c*-Z9R.

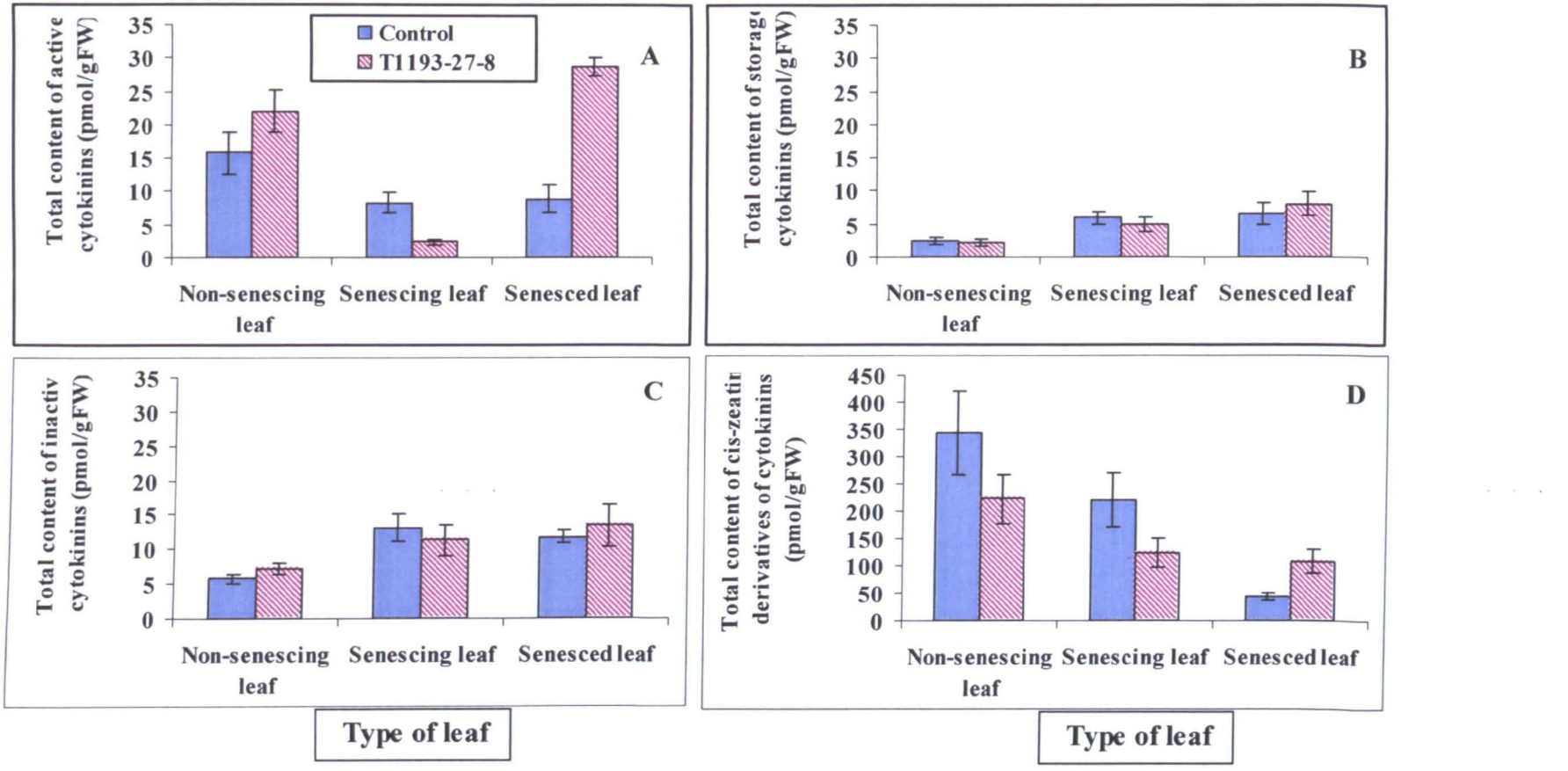

Figure 3.22. The total content of active (A), inactive (B), storage (C) and *cis*-zeatin and its derivative cytokinins (D) in the non-senescing (NS), senescing (SG) and senesced (SD) leaves of transgenic T1193-27-8 plant (striped bar) and control 1 plant (non-striped bar). Total content of active cytokinins = Z, DHZ, Z9R, DHZ9R, iP and iP9R; Total content of storage cytokinins = DHZOG, ZOG, Z9ROG and DHZ9ROG; Total content of inactive cytokinins = Z7G,Z9G, DHZ7G, DHZ9G, iP7G and iP9G; Total content of *cis*-zeatin and its derivatives = *c*-Z, *c*-Z7G, *c*-Z9G, *c*-ZOG, *c*-ZROG and *c*-Z9R.

fold higher in T1193-24-9 plant than in control plant. The levels of active CKs, storage CKs and *cis*-zeatin and its derivative CKs in the non-senescing and senescing leaves were similar in control and T1193-24-9 plants. The level of active CKs in the senesced leaf was lower in control plant than T1193-24-9 plant. On the otherhand, the levels of four groups of CKs in the three leaves were similar in control and T1193-27-8 plants (Figure 3.22). However, the level of *cis*-derivatives in the senesced leaf was higher in T1193-27-8 plant than in control plant. The level of active CKs in the senesced leaf was lower in the control plant than in T1193-27-8 plant.

3.4. Discussion

3.4.1 Integration of the genes in the transgenic T_0 rice plants

SAG12-*gusA*, SAG12-*ipt* and CAMV35S:*hpt* genes were introduced into the embryogenic calli, derived from mature rice seeds, using biolistic transformation. PCR analysis showed that the *gusA, ipt* and *hpt* genes were present in the hygromycin-resistant plantlets. Southern blot analysis was used to confirm the transgenic nature of the PCR-positive plants and to estimate the number of transgenes present. Southern blot analysis of the undigested DNA showed hybridization of the *gusA*, *SAG12-ipt-nos* and *hpt* probes to the DNA in the high molecular weight region which indicate integration of these genes into the genome. The digested DNA contained the fragment of the expected size, 1.87 kb for the *gusA* gene and 3.18 kb for the *SAG12-ipt-nos* gene. However, a high copy number of the *SAG12-ipt-nos* and *hpt* genes were observed in these transgenic rice plants. A high copy number of the *ipt* gene was also observed in transgenic wheat SAG12-*ipt* plants (Daskalova *et al.*, 2002). But a single copy of the *ipt* gene without rearragements in was observed in SAG12-*ipt* lettuce (McCabe, 2001). Gapper *et al.*, (2002) reported in many cases the integration of a single copy of the transgene in SAG12-*ipt* broccoli but Chen *et al.*, (2001) reported one to four restriction fragments of the *ipt* gene were found in the SAG12-*ipt* broccoli. Lin *et al.*, (2002) reported that more than 50% lines were integrated with a single copy

of the transgene and a majority of the lines contained 1-3 copies of the transgene in SAG12-*ipt* rice. Cao (2001) also reported that the integration with 1-3 copies of the transgenes, among which 42.29% were single copy of the transgene, in SA12-*ipt* rice.

3.4.2 Expression of the ipt gene in the leaves of transgenic T_0 rice plants

The SAG12 promoter, from *Arabidopsis thaliana,* should activate the expression of the *ipt* gene only at the start of leaf senescence Gan and Amasino (1995). The *ipt* gene was expressed in pre-senescing leaves of seven transformants, in senescing leaves of three transformants and in the senesced leaves of four transformants. The *ipt* gene was expressed in the senescing leaves of all the transgenic T_0 SAG12-*ipt* rice plants analysed. However, the *ipt* gene was also expressed even in the non-senescing leaves of transgenic plant Nos. 1194 and 2068 which indicate that the *ipt* gene was not properly driven by the senescence-specific promoter, SAG12, obtained from *Arabidopsis thaliana.* The promoter from dicot may not have worked well in a monocot like rice. The leaky expression of the *ipt* gene was also observed in SAG12-*ipt Petunia* plants (H. Klee., personal communication). Gapper *et al.,* (2002) also reported that some of the SAG12-ipt lines of broccoli showed signs of SAG12 promoter leakiness. The leaky expression of another promoter, heat shock promoter, fused the *ipt* gene was also observed when the *ipt* gene was expressed in the leaves of plants at 20°C (Smart *et al.,* 1991).

3.4.3 Cytokinin content in the leaves of transgenic T_0 rice plants

Genetic manipulation of the *ipt* gene affects the production of cytokinins in the leaves of the transgenic plants. In this study, the levels of active cytokinins in the senesced leaves of plant No. T1193 and their T_2 progeny were higher in the leaves of the transgenic T_0 rice plants compared to the untransformed control plants. The zeatin-type of CKs was also the major form of active CKs produced on heat shock (Medford *et al.,* 1989; Smart *et al.,* 1991).

3.4.4 Phenotype of the transgenic T_0 plants

Delayed leaf senescence was not always obvious in the transgenic T_0 SAG12-*ipt* rice plants obtained in this study because the plants were at different stages of growth due to variation in the time of shoot regeneration from inoculated plants. This observation was also reported in transgenic T_0 SAG12-*ipt* lettuce plants (McCabe *et al.*, 2001). In this study, delayed leaf senescence, delayed flowering by 2-4 weeks, wrinkled leaves, shorter plant height was observed in four T_1 progeny of plant No. 1193 which might be due to the expression of the *ipt* gene and production of cytokinins. The delay in leaf senescence was due to the high levels of active cytokinins present in the senesced leaves of the T_2 progeny of Plant No. 1193. These results are consistent with the previous findings. Hewelt *et al.*, (1994) reported that the tobacco plants expressing the *ipt* gene showed a four-week delay in flowering, stunted plant height, and reduced fertility. These alterations in the phenotypes are due to the increased levels of cytokinins. Estruch *et al.*, (1993) reported that the alterations of floral development and no seed production were observed in situations wherein the cytokinin level was increased by 500-fold. Smigocki (1995) reported that a few tobacco plants containing a wound-inducible proteinase-inhibitor-IIK gene promoter fused to the *ipt* gene (PI-II-*ipt*) failed to flower but most of the PI-II-*ipt* plants showed a 2-week delay in flowering, wrinkled leaves, a dark green color of leaves, delayed leaf senescence, shorter (20-50%) compared to control plants, decreased pod number per plant from about 100 on control plants to fewer than 20 on PI-II-*ipt* plants, and fewer seeds per pod. McKenzie *et al.*, (1998) also observed that there was delayed leaf senescence and increased concentration in one line of tabacco with Cu-controllable promoter fused to the *ipt* gene. Transformed tobacco plants containing the soybean heatshock promoter fused to the *ipt* gene were also shorter and showed delayed leaf senescence compared to the control plants (Medford *et al.*, 1989; Smart *et al.*, 1991). Chen *et al.*, (2001) observed some phenotypic or morphological alterations in transgenic SAG12-*ipt* broccoli. Gapper *et al.*, (2002)

also reported that some of the transgenic SAG12-*ipt* lines of broccoli showed phenotype changes such as reduced or no roots and reduced plant stature which are typical of constitutive expression of the *ipt* gene (McKenzie *et al.*, 1998). McCabe *et al.*, (2001) also reported that the T_2 lines of transgenic SAG12-*ipt* lettuce showed delayed in bolting, leaf senescence, flower bud formation and panicle development and the transgenic plants required 4 to 8 weeks longer to produce flower and seeds. However, Gan and Amasino (1995) obtained transgenic SAG12-*ipt* tobacco plants with delayed leaf senescence, high seed yield but without any changes in the plant morphology. Cao, (2001) and Lin *et al.*, (2002) produced transgenic SAG12-*ipt* rice plants with delayed leaf senescence, increased grain filling percentage. However, Lin *et al.*, (2002) observed that the SAG12-*ipt* rice plants had a lower plant height compared to the control plants.

Chapter 4. Isolation of the homologue of the *Arabidopsis* SAG12 gene in NPT rice

4.1 Introduction

The *SAG12* gene is expressed in the senescent leaves of *Arabidopsis thaliana* (Noh and Amasino, 1999). The *SAG12* promoter, fused to the *isopentenyl transferase (ipt)* gene, allowed the expression of the *ipt* gene in the senescing leaves of the transgenic tobacco plants and delayed leaf senescence in tobacco (Gan and Amasino, 1995) and in rice (Cao, 2001; Lin *et al.*, 2002). However, the results in Chapter 3 showed that a leaky expression was observed in some transgenic rice plants transformed with SAG12-*ipt* construct. This suggested that the SAG12 promoter from the dicot did not work well in the monocot background. Hence, it was decided to isolate a promoter from rice that could replace the SAG12 promoter.

Two strategies were adopted in the isolation of the rice homologue of the SAG12 promoter. The first strategy involved the expression of the *SAG12* gene in rice whereas the second strategy involved the expression of the other genes which are involved in leaf senescence. The SAG12 encodes cysteine protease. The first strategy involves the search for sequences of cysteine proteases in other monocots which have homology with the amino acid sequence of SAG12. A phylogenetic tree is constructed to determine if rice cysteine protease clustered closest to SAG12. PCR primers is designed in these rice BAC clones and RT-PCR analysis is done to determine its expression in different parts of the plant. The clones which have homology with the senescing rice will be cloned into a vector, transformed into *E.coli* and digested with appropriate enzymes. The sequence of the clones is obtained by sequencing. The sequence of these clones will enable the design of primers and these clones will be used further in the isolation of the rice homologue. The second strategy involves expression of several senescence related genes in different parts of the rice plant. Those clones which are highly expressed is used to find the homology of this cysteine protease in rice.

4.2. Materials and Methods

4.2.1 Tblastn search

Tblastn was used to probe and search various databases with the amino acid sequence of the *Arabidopsis' SAG 12* gene (GenBank accession number AAC49135). The databases used were the National Center for Biotechnology Information (NCBI), DNA Data Bank of Japan (DDBJ) and Monsanto databases with the following website addresses: http://www.ncbi.nil.nih.gov, http://srs.ddbj.nig.ac.jp and http://www.rice-research.org, respectively. A phylogenetic tree was constructed for *SAG12*-related genes using the HMGRP program with the website address: http://www.hgmp.mrc.ac.uk. PCR primers were designed from the coding region of two rice BAC clones, namely, OSM146118 and OSM146316, which clustered closest and had a high homology with the amino acid of the *Arabidopsis' SAG12* gene.

4.2.2 RNA extraction and cDNA synthesis

The total RNA was extracted from various parts of the rice plant, namely, pre-senescing leaf, senescing leaf, senesced leaf, roots, stems, leaf sheaths, panicle with flowers and panicle with mature grains using the SV Total RNA Isolation System (Promega). To ensure that the RNA was the real template, the extracted RNA was DNAse-treated with DNAse (Promega) to remove any DNA contamination. Using the PowerScript Reverse Transcriptase (Clontech), the cDNA was synthesized from 2.5 µg of total RNA extracted from each plant tissue.

4.2.3 RT-PCR analysis

RT-PCR analysis was done using the following reaction mixture: 15.4 µl of sterile MQ water, 2.5 µl of 10X PCR buffer, 2.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10mM dNTPs, 0.5 ul of 10 µM forward primer, 0.5 µl of 10 µM reverse primer, 3 µl of

cDNA and 0.1 µl of PFU polymerase or Taq polymerase. The sequences of primers used in RT-PCR analysis are shown in Table 4.1. The following RT-PCR profile was used: 1 cycle of 95°C for 2 minutes, 40 cycles of 95°C for 1 minute, 65°C for 1 minute and 72°C for 2 minutes and 1 cycle of 72°C for 5 minutes. The RT-PCR products were analyzed by running them in 1.2 % agarose gel electrophoresis.

4.2.4 Ligation of the eluted RT-PCR product into the cloning vector and Transformation of *E. coli*

The RT-PCR products, obtained using OSM146316 primers, in the pre-senescing (P), senescing (SG) and senesced (SD) leaves in 1%(w/v) agarose gel were excised and eluted from the excised agarose gel using Bio101 kit (Analchem). The eluted RT-PCR products were ligated and inserted into the pPCR-Script Amp SK(+) Cloning vector (Stratagene). Transformation of *E. coli* was done using the XL10-Gold ultracompetent cells (Stratagene) and spread on an LB-Amp agar plate. Twenty four white colonies were obtained for each P, SG, and SD transformation.

4.2.5 Isolation, Purification and Digestion of Plasmid DNA

Twenty four single white colonies were obtained for each of the pre-senescing clones (labelled P), senescing clones (labelled SG), and senesced clones (labelled SD). Each selected colony was grown in an LB-Amp liquid medium. The plasmid DNA of the P, SG and SD clones (P = pre-senescing clones, SG = senescing clones, SD = senesced clones) were isolated from *E. coli* using the Wizard Plus Miniprep DNA Purification System (Promega). The overnight grown 5 ml bacterial cultures were centrifuged at 13,000 rpm for 5 minutes. The supernatant was discarded and the pellet was air dried. The pellet was resuspended in 300 µl resuspension buffer, the cells were lysed with 300 µl lysis buffer and then neutralised with 300 µl neutralisation buffer. The mixture was centrifuged for 5 minutes at 13,000 rpm and the supernatant was transferred to a new tube. One ml

of DNA binding resin was added to the supernatant which was applied to a mini-column. The columns were washed with 3 ml of wash solution and purified DNA was eluted from the column with 100 µl of hot sterile water. The purified plasmid DNA was digested with *KpnI* and *SacI* at 37°C overnight and ran on a 1.2% agarose gel at 70 volts for an hour. The digested plasmid DNA of the pre-senescing (P), senescing (SG) and senesced (SD) clones showed an insert size of the expected size.

4.2.6 Sequencing of several P, SG and SD clones

The plasmid DNA of several P, SG and SD clones, containing the insert, were sent for sequencing to the MWG- AG BIOTECH company in Germany.

4.2.7 Blastn and blastx searches of several P, SG and SD clones

Blastn and blastx searches were made for the sequences of two P clones, five SG clones and two SD clones by submitting the sequence of the clones to the http://www.ncbi.nil.nih.gov and using the NCBI database. The sequence of the SG8 clone was submitted to the Monsanto database to find the rice BAC clones which would have some homology to it. Primers were designed from this rice BAC clone RT-PCR analysis of the OSM13394 rice clone was done using all parts of the rice plant.

To identify possible rice promoters that could replace *Arabidopsis* SAG12, a database search of the other genes involved in leaf senescence was made. The senescence-associated and senescence related genes found are *the SAG 101, ORE9, red chlorophyll catabolite (RCC) and chlorophyllase.* A Tblastn search of these genes was made using the Monsanto database. PCR primers were designed for these genes. RT-PCR analysis of these rice BAC clones was done using the cDNA from the pre-senescing leaf, senescing leaf, senesced leaf, roots, stems, leaf sheaths, panicle with flowers and panicle with mature grains of the rice plant.

Table 4.1. Sequence of primers used in RT-PCR analysis of the *SAG12* and senescence related genes in various parts of NPT rice.

Gene	Type of Primer	Sequence of Primer
OSM146118	Forward	5' CCT AGG ACC TCA AAG GTG TTG C 3'
OSM146118	Reverse	5' CCA CAT CAC ACT TGA GCT TTC CC 3'
OSM146316	Forward	5' CCC CAC CCC AGG AGC AGA AGT GC 3'
OSM146316	Reverse	5' CGT ACG CTA GCT TCG TAG TAA G 3'
OSM1359	Forward	5' CCA TGG GTT GAT TAC TCT GGC AG 3'
OSM1359	Reverse	5' CAT GCT TGG ACT CCA CGG ATA GG 3'
OSM 1366	Forward	5' GGA CAA GCT GAT GGC GCT GG 3'
OSM 1366	Reverse	5' GAT GTA CTT GGG CAG TCG GAG 3'
OSM13394	Forward	5' GAG AAG CAG AAG TAC TCC AAC C 3'
OSM13394	Reverse	5' CCT CGT CGA ACA TGT CGA CGA A 3'
OSM 141989	Forward	5' CAA CAG CAG CGG TTC AGA TGC 3'
OSM 141989	Reverse	5' GTC GGC AAT GTC AAG TGG CTC 3'
OSM 143080	Forward	5' ACT TCT ACG TGC GGA GCA TC 3'
OSM 143080	Reverse	5' AAC ACC AGC CCA ACT CCG GCG AA 3'
OSM 151086	Forward	5' CTC GAC CTC TCA CTT GTC TC 3'
OSM 151086	Reverse	5' GAG GTT GGG TGG GCATTG GT 3'
OLIGODT	Forward	5' TTT TTT TTT TTT TTT TTT TTTTTTTVN3'

The 0.339 kb transcript OSM1282 rice BAC clone, with homology to the chlorophyllase gene, was cloned into pGEM-T Easy vector. The plasmid DNA was digested with *EcoRI* and sent to MWG for sequencing. The 0.35 kb transcript, obtained in the senescing leaf after RT-PCR analysis of the OSM1282 clone, has been cloned into pGEM-T Easy vector and labelled as a C clone and this C clone was sent to MWG-AG BIOTECH for sequencing. Blastn and blastx searches of this C clone were made using the NCBI database.The 0.339 kb transcript (or RT-PCR product) was excised from a 1% (w/v) agarose gel and eluted using the Geneclean system according to the manufacturer's instructions (BIO I0I: Anachem, Beds, UK). The eluted RT-PCR product was ligated into the pGEM-T Easy vector (Promega) using the manufacturer instructions. The ligation reaction was set up as follows:

Vector	1 μl
Insert	1 μl (neat or 1/10 dilution)
10X buffer	1 μl
Ligase	1 μl
Water	6 μl

The ligation reaction was left at 4 °C overnight.

An overnight culture in LB was diluted 100 fold into fresh LB and grown for 4 hours. The culture was spun down at 3,000 rpm for 5 minutes and was washed 3 times in 10% (v/v) glycerol. The resulting reaction mix was diluted in 500 μl LB, grown for 1 hour at 37°C and plated out onto LB agar plates with 50 μl/ml ampicillin and X-gal/IPTG. Transformation was done using the XL10-Gold ultracompetent cells (Stratagene) and plated out onto LB agar plates with 50 μl/ml ampicillin and X-gal /IPTG. Twenty four white colonies were obtained for each ligation reaction or P, SG, and SD transformation were selected and grown overnight at 37 °C in LB containing 50 μg/ml ampicillin. The plasmid DNA was isolated and purified as described earlier using (Wizard miniprep-Promega) and digested with *Eco*RI to confirm the presence of inserts. Restriction enzyme digests consisted of:

DNA	40 μl
*Eco*RI	1 μl
10X Buffer	6 μl
Water	11 μl

The reaction was left overnight at 37 °C water bath. The digestion products were separated on a 1% (w/v) agarose gel and inserts of the correct size (when compared with the original PCR products) were excised and purified using the Geneclean system (BIO I0I: Anachem, Beds, UK).

4.3. Results

4.3.1. Expression of the SAG12 gene in rice

The *SAG12* gene encodes a cysteine protease in *Arabidopsis thaliana.* The Tblastn search using the SAG12 protein (GenBank accession number AAC49135), in Figure 4.1, showed that it had some homology with the cysteine proteases of rape and several monocots such as rice, barley and *Zea mays* (Table 4.2). A phylogenetic tree of the *SAG12* gene showed that two rice BAC clones, OSM146118 and OSM146316, clustered closest to it (Figure 4.2). A blastn search of the Monsanto database showed that these two rice BAC clones had a high homology with the *Arabidopsis' SAG12* gene (U37336). PCR primers were designed from the coding region of these two rice BAC clones.

MALKHMQIFLFVAIFSSFCFSITLSRPLDNELIMQKRHIEWMTKHGRVYAD
VKEENNRYVVFKNNVERIEHLNSIPAGRTFKLAVNQFADLTNDEFCSMYT
GFKGVSALSSQSQTKMSPFRYQNVSSGALPVSVDWRKKGAVTPIKNQGS
CGCCWAFSAVAAIEGATQIKKGKLISLSEQQLVDCDTNDFGCEGGLMDT
AFEHIKATGGLTTESDYPYKGEDATCNSKKTNPKATSITGYEDVPVNDEQ
ALMKAVAHQPVSVGIEGGGFDFQFYSSGVFTGECTTYLDHAVTAIGYGES
TNGSKYWIIKNSWGTKWGESGYMRIQKDVKDKQGL CGLAMKASYPTI

Figure 4.1. The sequence of the *Arabidopsis thaliana*'s *SAG12* protein (GenBank accession number AAC49135)

Table 4.2. Tblastn results of the *Arabidopsis SAG12* gene using the NCBI and DDBJ databases.

GenBank Accession Number	Definition	Source	E value	Reference
AAC49135	senescence-specific protein	*Arabidopsis thaliana*	-112	Gan and Amasino (1995)
AF019147	cysteine proteinase Mir3	*Zea mays*	-91	Pechan *et al.*, (1999)
AB020961	cysteine protease component of protease-inhibitor	*Zea mays*	-91	Yamada (2000)
X80876	cysteine protease	*Oryza sativa*	-90	Shintani *et al.*, (1995)
Z97023	cysteine endopeptidase EP-A	*Hordeum vulgare*	-88	Lok and Sorensen Unpublished
U94591	*Hordeum vulgare* cysteine proteinase precursor, mRNA	*Hordeum vulgare*	-88	Porali *et al.*, Unpublished
D90406	oryzain alpha	*Oryza sativa*	-87	Watanabe (1991)
AF191027	papain-type cysteine endopeptidase XCP1 mRNA	*Arabidopsis thaliana*	-86	Zhao *et al.*, (2000)

AF191028	papain-type cysteine endopeptidase XCP2 mRNA	*Arabidopsis thaliana*	-84	Zhao *et al.*, (2000)
AF099203	cysteine endopeptidase precursor (EP3A) gene	*Oryza sativa*	-79	Ho *et al.*, (2000)
U19384	cysteine proteinase EPB2 gene	*Hordeum vulgare*	-78	Koehler and Ho (1990)
U19359	cysteine proteinase EPB1 gene	*Hordeum vulgare*	-78	Koehler and Ho (1990)
AF019145	cysteine protease Mir1	*Zea mays*	-74	Pechan (1999)
AF019146	cysteine proteinase Mir2	*Zea mays*	-73	Pechan (1999)
Z97022	cysteine proteinase	*Hordeum vulgare*	-101	Lok and Sorensen Unpublished
AF089849	senescence-specific cysteine protease	*Brassica napus*	-101	Noh and Amasino (1999)
AF089848	senescence-specific cysteine protease	*Brassica napus*	-101	Noh and Amasino (1999)

OSM146118	rice BAC clone	*Oryza sativa*	-100	Monsanto database
OSM146316	rice BAC clone	*Oryza sativa*	-99	Monsanto database
OSM13176	rice BAC clone	*Oryza sativa*	-82	Monsanto database
D90407	oryzain beta	*Oryza sativa*	-81	Watanabe *et al.*, (1991)
AB004819	Rep 1 cysteine endopeptidase	*Oryza sativa*	-80	Kato *et al.*, (1999)

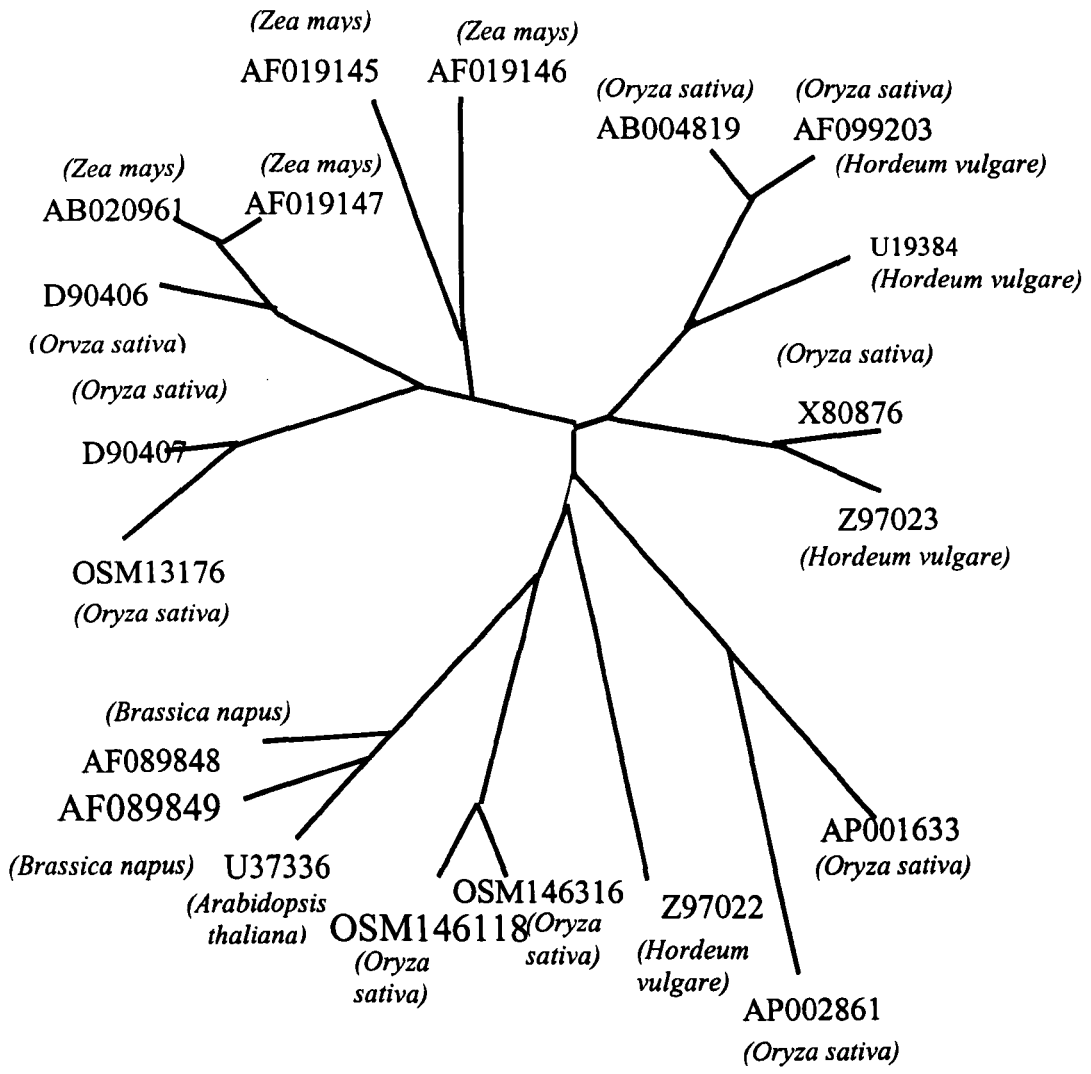

Figure 4.2. Phylogenetic tree of the *Arabidopsis thaliana*'s *SAG12 gene* (Accession number U37336).

The glyceraldehyde-3-phosphate dehydrogenase (*g3pdh)* gene, expressed in all parts of the rice plant, contains an intron. As a control, RT-PCR analysis of the *g3pdh* gene will give a 0.36 kb fragment using the RNA template and a 0.65 kb fragment in the presence of DNA. RT-PCR analysis for the *g3pdh* gene showed the absence of a 0.65 kb fragment (Figure 4.3, lanes 1-6) which indicate that the DNAse-treated RNA was not contaminated with DNA, hence, the RT-PCR analysis using the cDNAs from various parts of the rice plant and the primers of the OSM146118 and OSM146316 rice BAC clones would give a reliable result.

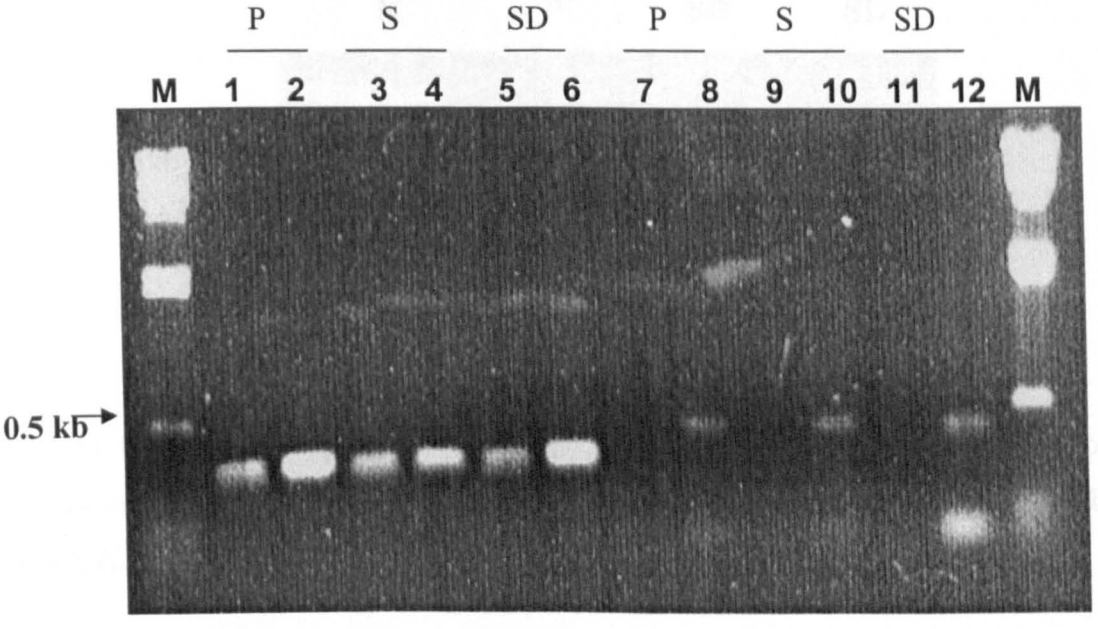

Figure 4.3. RT-PCR analysis of the glyceraldehyde-3-phosphate dehydrogenase (*g3pdh)* gene (lanes 1-6) and the OSM 146118 rice BAC clone (lanes 7-12) in the pre-senescing (P), senescing (S) and senesced (SD) leaves of NPT rice. Lanes 1, 3, 5, 7, 9, and 11 were amplified with 30 cycles while lanes 2, 4, 6, 8, 10 and 12 were amplified with 40 cycles. M = lambda DNA/*Hind III* marker.

RT-PCR analysis of the OSM146118 rice BAC clone showed a 0.49 kb transcript of equal intensity in the pre-senescing, senescing and senesced leaves of rice which indicate that it is not senescence-specific (Figure 4.3, lanes 8, 10 and 12).

RT-PCR analysis for the OSM146316 clone showed 0.18 kb, 0.334 kb and 0.339 kb transcripts in the pre-senescing, senescing and senesced leaves, respectively (Figure 4.4). The digested plasmid DNA of the pre-senescing, senescing and senesced clones showed an insert size which is the same size as those bands obtained in Figure 4.4.

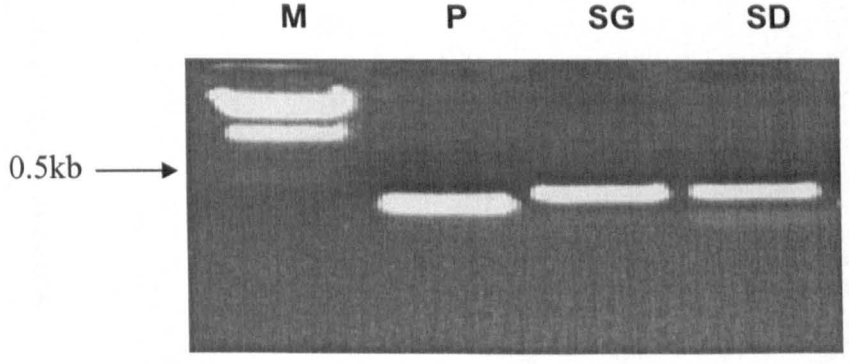

Figure 4.4. RT-PCR analysis of the OSM146316 rice BAC clone in the pre-senescing (P), senescing (SG) and senesced (SD) leaves of a rice plant. M= lambda DNA/*Hind III* marker.

The sequencing results of the two P clones (labelled P1 and P2) obtained from the pre-senescing leaf, five SG clones (labelled SG8, SG9, SG10, SG11 and SG12) obtained from the senescing leaf, and two SD clones (labelled as SD3 and SD4) obtained from the senesced leaf are shown in Table 4.3. A blastn search of the sequences of P, SG and SD clones was made using the NCBI database. The blastn search result showed that only 18 base pairs of P1 clone and 19 base pairs of P2 clone were homologous with *Arabidopsis thaliana* BAC clones which indicate low homology. On the other hand, blastn search of SG8, SG9, SG10, SG11,SG12, SD3 and SD4 clones showed 175 to 311 base pairs homology with rice BAC clones (Table 4.4). Blastx search of SG8, SG9, SG10, SG11,SG12, SD3 and SD4

Table 4.3. Sequencing results of the clones obtained from pre-senescing, senescing and senesced rice leaves.

Clone	Source of the clone	Sequence of the clone
P1	pre-senescing leaf	CTTACTACGAAGCTAGCGTACGTCTAACACGGCGTCAACACAGCAAACAACCGGGACAGACAGGGCA ACAATATACAAAAGAACCCTAGGGTGAATCAAACCAAAAAAAAAAAAAAGAGTGGATAAGACGCAAA CGAGACGAAAAAAAAAAGATGTCCACTTCTGCTCCTGGGGTGGGGGGG
P2	pre-senescing leaf	CTTACTACGAAGCTAGCGTACGTCTAACACGGCGTCAACACAGCAAACAACCGGGACAGACAGGGCA ACAATATACAAAAGAACCCTAGGGTGAATCAAACCAAAAAAAAAAAAAGAGTG
SG8	senescing leaf	CTTACTACGAAGCTAGCGTACGTCGTGGCGTCCTCGTCGAACATGTCGACGAAGTAGCCCTCTCGCAG GCCGAGCAGCCTGGCCAATCTGGCGAGCACAAGGCTGGTGATCTCCCTGCACCTCATAGTGTACTCAT GCAGAACATCTCTGAAAGAAGGAGGCTGTGTTGGCCAGAGGCTATGGGCTATCCGGGACTCAGGCTC AACGATGTGGTAGAACCGGTCGCACCAGTTCAGGATCTGTTTCTCTGACACGACCATGTCGTTGCCGT ACCCTTCGATCCTGAAATCCTTGCCGTTCACCAGGTTGGAGTACTTCTGCTCCTGGGGTGGGG
SG9	senescing leaf	CTTACTACGAAGCTAGCGTACGTCGTGGCGTCCTCGTCGAACATGTCGACGAAGTAGCCCTCTCGCAG GCCGAGCAGCCTGGCCAATCTGGCGAGCACAAGGCTGGTGATCTCCCTGCACCTCATAGTGTACTCAT GCAGAACATCTCTGAAAGAAGGAGGCTGTGTTGGCCAGAGGCTATGGGCTATCCGGGACTCAGGCTC AACGATGTGGTAGAACCGGTCGCACCAGTTCAGGATCTGTTTCTCTGACACGACCATGTCGTTGCCGT ACCCTTCGATCCTGAAATCCTTGCCGTTCACCAGGTTGGAGTACTTCTGCTCCTGGGGTGGGG
SG10	senescing leaf	CCCCACCCCAGGAGCAGAAGTTGGCAACGCAGGTGAATCACCAGAAACATCCATCTCATCATGTGAA ACATGTGGAGGTGAACTAGGAGGGCTCGAAACAGACAGAGAAGATTGTGAGCCCAATATAGGGTTTT

		CCCTATCCCCATTTTGCTGTTCATCGAGACCAATGCCTTCAGCTGATGTCCCATGCTGGTGAGATGCCT CTTGGTATACACGTCCATATTCGGTCTGCACAGCATGCTCTTCCCTACTAGAACTCTCTCTCAACCCAC TTTCCACAAAAGAAGAATTCGCATGTTGAGTGGAAGGACCAACCGTACTTCTGCTCCTGGGGTGGGG
SG11	senescing leaf	CCCCACCCCAGGAGCAGAAGTACTCCAACCTGGTGAACGGCAAGGATTTCAGGATCGAAGGGTACGG CAACGACATGGTCGTGTCAGAGAAACAGATCCTGCACTGGTGCGACCGGTTCTACCACATCGTTGAGC CTGAGTCCCGGATAGCCCATAGCCTCTGGCCAACACAGCCTCCTTCTTTCAGAGATGTTCTGCATGAG TGCACTATGAGGTGCAGGGAGATCACCAGCCTTGTGCTCGCCAGATTGGCCAGGCTGCTCGGCCTGCG AGAGGGCTACTTCGTCGACATGTTCGACGAGGACGCCACGACGTACGCTAGCTTCGTAGTAAG
SG12	senescing leaf	CCCCACCCCAGGAGCAGAAGTACTCCAACCTGGTGAACGGCAAGGATTTCAGGATCGAAGGGTACGG CAACGACATGGTCGTGTCAGAGAAACAGATCCTGAACTGGTGCGACCGGTTCTACCACATCGTTGAGC CTGAGTCCCGGATAGCCCATAGCCTCTGGCCAACACAGCCTCCTTCTTTCAGAGATGTTCTGCATGAG TACACTATGAGGTGCAGGGAGATCACCAGCCTTGTGCTCGCCAGATTGGCCAGGCTGCTCGGCCTGCG AGAGGGCTACTTCGTCGACATGTTCGACGAGGACGCCACGACGTACGCTAGCTTCGTAGTAAG
SD3	senesced leaf	CCCCCCCACCCCAGGAGCAGAAGTTGGCAACGCAGGTGAATCACCAGAAACATCCATCTCATCATGT GAAACATGTGGAGGTGAACTAGGAGGGCTCGAAACAGACAGAGAAGATTGTGAGCCCAATATAGGG TTTTCCCCATCCCCATTTTGCTGTTCATCGAGACCAATGCCTTCAGCTGATGTCCCATGCTGGTGAGAT GCCTCTTGGTATACACGTCCATATTCGGTCTGCATAGTATGCTCTTCCCTACTAGAACTCTCTCTCAAC CCACTTTCCACAAAAGAAGAATTCGCATGTTGAGTGGAAGGACCAACCGTACTTCTGCTCCTGGGGTG GGGGGGC
SD4	senesced leaf	CCCCACCCCAGGAGCAGAAGTTGGCAACGCAGGTGAATCACCAGAAACATCCATCTCATCATGTGAA ACATGTGGAGGTGAACTAGGAGGGCTCGAAACAGACAGAGAAGATTGTGAGCCCAATATAGGGTTTT CCCTATCCCCATTTTGCTGTTCATCGAGACCAATGCCTTCAGCTGATGTCCCATGCTGGTGAGATGCCT CTTGGTATACACGTCCATATTCGGTCTGCATAGTATGCTCTTCCCTACTAGAACTCTCTCTCAACCCAC TTTCCACAAAAGAAGAATTCGCATGTTGAGTGGAAGGACCAACCGTACTTCTGCTCCTGGGGTGGGG

Table 4.4. Blastn results of the clones obtained from pre-senescing, senescing and senesced rice leaves.

Clone	Source of the clone	Blastn results			
		GenBank Accession Number	**The clone is homologous with**	**Percentage homology (at DNA level)**	**Source**
P1	pre-senescing leaf	AC004473	chromosome 1 BAC T13D8	18/18=100%	*Arabidopsis thaliana*
P2	pre-senescing leaf	AC004473	chromosome 1 BAC T13D8	19/19=100%	*Arabidopsis thaliana*
SG8	senescing leaf	AP003019	Genomic DNA, Chromosome 6, BAC clone	175/175=100%	*Oryza sativa*
SG9	senescing leaf	AP003019	Genomic DNA, Chromosome 6, BAC clone	174/175=99%	*Oryza sativa*
SG10	senescing leaf	AC079853	complete sequence	309/313=98%	*Oryza sativa*
SG11	senescing leaf	AP003019	Genomic DNA, Chromosome 6, BAC clone	174/175=99%	*Oryza sativa*

SG12	senescing leaf	AP003019	Genomic DNA, Chromosome 6, BAC clone	175/175=100%	*Oryza sativa*
SD3	senesced leaf	AC079853	Complete sequence	310/313=99%	***Oryza sativa***
SD4	senesced leaf	AC079853	Complete sequence	311/313=99%	*Oryza sativa*

Table 4.5 Blastx results of the clones obtained from pre-senescing, senescing and senesced rice leaves.

Clone	**Source of the clone**	**Blastx results**			
		GenBank Accession Number	**Clone is homologous with**	**Percentage homology (at protein level)**	**Source**
P1	pre-senescing leaf	AAC08053	60K inner-membrane protein homolog	14/35=40%	*Treponema pallidum*
P2	pre-senescing leaf	No significant similarity found	No significant similarity found	No significant similarity found	No significant similarity found
SG8	senescing leaf	BAA95829	Similar to *Prunus armeniaca* ethylene-forming-enzyme-dioxygenase, (U97530) *[Oryza sativa]*	75/106= 70%	*Prunus armeniaca*
SG9	senescing leaf	BAA95829	Similar to *Prunus armeniaca* ethylene-forming-enzyme-dioxygenase,	75/106= 70%	*Prunus armeniaca*

			(U97530) ***[Oryza sativa]***		
SG10	senescing leaf	AAK52550	Unknown protein (*Oryza sativa*)	74/111= 66%	*Oryza sativa*
SG11	senescing leaf	*BAA95829* (AP002069)	Similar to *Prunus armeniaca* ethylene-forming-enzyme- dioxygenase (U97530) [*Oryza sativa*]	72/106= 67%	*Prunus armeniaca*
SG12	senescing leaf	BAA95829	Similar to *Prunus armeniaca* ethylene-forming-enzyme- dioxygenase. (U97530) [*Oryza sativa*]	74/106= 69%	*Prunus armeniaca*
SD3	senesced leaf	AAK52550	*Unknown protein, Oryza sativa*	76/112= 67%	*Oryza sativa*
SD4	senesced leaf	AAK52550	*Unknown protein,Oryza sativa*	76/112= 68%	*Oryza sativa*

clones showed that 72 to 76 amino acids were homologous to *Oryza sativa* or *Prunus armeniaca* (Table 4.5). The alignment of senescing SG8 clone sequence with the *Prunus armeniaca's* ethylene-forming-enzyme-dioxygenase was shown in Figure 4.5. It was noted that when the GenBank accession number BAA95829 was accessed, the Prunus armeniaca's ethylene-forming-enzyme-dioxygenase was expressed in ripe fruit and not in senescing leaf of apricot.

```
Query:326QEQKYSNLVNGKDFRIEGYGNDMVVSEKQILNWCDRFYHIVEPESRIAHSLWPTQPPSFR 147
          ++QKYSNLVNGKDFRIEGYGNDMVVSEKQILNWCDRFYHIVEPE
Sbjct:117EKQKYSNLVNGKDFRIEGYGNDMVVSEKQILNWCDRFYHIVEPE---------------- 160

Query: 146 DVLHEYTMRCREITSXXXXXXXXXXXXREGYFVDMFDEDATTYASF 9
           DVLHEYTMRCREITS            REGYFVDMFDEDATTYA F
Sbjct: 161 DVLHEYTMRCREITSLVLARLARLLGLREGYFVDMFDEDATTYARF 206
```

Figure 4.5. Alignment of senescing SG8 clone sequence with the *Prunus armeniaca's* ethylene-forming-enzyme-dioxygenase.

The senescing SG8 clone had 70% homology (75/106 amino acids) with the *Prunus armeniaca's* ethylene-forming-enzyme-dioxygenase. A Tblastn search of the senescing SG8 clone sequence showed that it was homologous with OSM 13394 rice BAC clone. Primers were designed from this rice BAC clone to amplify a 0.22 kb fragment.

RT-PCR analysis of the OSM13394 rice clone showed the absence of a 0.25 kb transcript in the stem and the presence of a strong and equal intensity of 0.25 kb transcript in all parts of the rice plant except the stem (Figure 4.6), hence, this gene is not senescence-specific. Since the OSM13394 rice clone is not senescence-specific, efforts were made to isolate a promoter from the senescence-related genes.

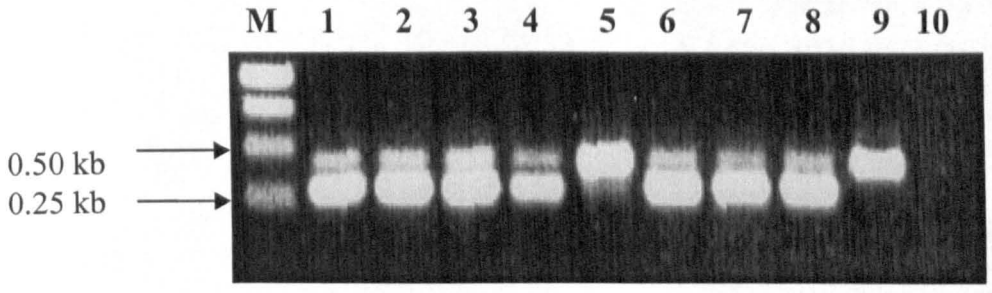

Figure 4.6. RT-PCR analysis of the OSM13394 rice BAC clone in various parts of NPT rice. lane 1 = pre-senescing leaf; lane 2 = senescing leaf; lane 3 = senesced leaf; lane 4 = roots; lane 5 = stem; lane 6 = leaf sheath; lane 7 = panicle with flowers; lane 8 = panicle with mature grains; lane 9 = DNA; lane 10 = water; M = 1 kb DNA ladder.

4.3.2. Expression of the senescence-related genes in rice

To identify possible rice promoters that could replace *Arabidopsis* SAG12, a database search of the other genes involved in leaf senescence was made. The senescence-associated and senescence related genes found were *SAG 101, ORE9, red chlorophyll catabolite (RCC) and chlorophyllase*. A Tblastn search of these genes, made using the Monsanto database, showed that these genes had some homology with the rice BAC clones (Table 4.6).

SAG101 gene is not expressed in expanding leaves and in fully expanded pre-senescing leaves. *SAG101* gene had some homology with OSM14989 rice BAC clone (Table 4.6). RT-PCR analysis of the OSM14989 clone, labelled as SAG101 in Figure 4.7A, showed a transcript of the expected size, 0.60 kb, in all the plant parts, with highest intensity in the senescing leaf, senesced leaf, stem and leaf sheath and high intensity in the pre-senescing leaf, roots, panicle with flowers and panicle with mature grains (Table 4.7).

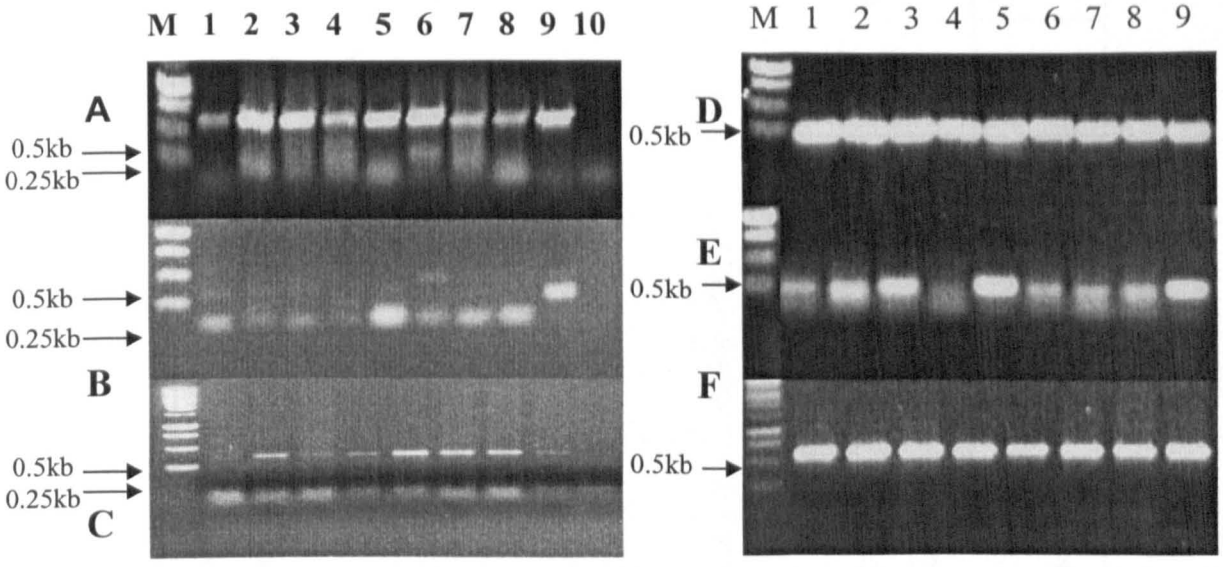

Figure 4.7. RT-PCR analysis of senescence-associated and senescence-related genes in various parts of NPT rice. A = *SAG101* gene ; B = *ORE9-1* gene; C = *chlorophyllase* gene; D = *ORE9-2* gene; E = *YLS3* gene; F = *RCC* gene; lane 1 = pre-senescing leaf; lane 2 = senescing leaf; lane 3 = senesced leaf; lane 4 = roots; lane 5 = stem; lane 6 = leaf sheath; lane 7 = panicle with flowers; lane 8 = panicle with mature grains; lane 9 = DNA; lane 10 = water; M = 1 kb DNA ladder.

ORE9 gene had some homology with OSM1359 and OSM151086 rice BAC clones (Table 4.6). RT-PCR analysis of the OSM 1359 clone, labelled as ORE9-1 in Figure 4.7B, was expressed in equal intensity in all the plant parts (Table 4.7). RT-PCR analysis of the OSM151086 clone, labelled as ORE9-2 in Figure 4.7D, showed a faint 0.30 kb transcript in the pre-senescing leaf, senescing leaf and senesced leaf and a faint 0.5 kb transcript in the leaf sheaths (Table 4.7). *YLS 3* gene had some homology with OSM1366 rice BAC clone (Table 4.6). RT-PCR analysis of the OSM1366 clone, labelled as YLS3 in Figure 4.7E, showed a 0.25 kb transcript in all plant parts except the roots (Table 4.7). A very robust 0.25 kb band was observed in the senescing leaf, senesced leaf and stem. *RCC* gene from barley had some homology with OSM128710 rice BAC clone (Table 4.6) which was expressed in equal intensity in all the plant parts (Figure 4.7F; Table 4.7).

Table 4.6. TBlastn result of senescence-associated and senescence-related genes.

Gene or Clone	GenBank Accession Number	Gene encodes for a	Reference	Source of the Gene	Blastx result		
					Rice BAC clone	Score (bits)	E-value
SAG 101	AF239888	hypothetical protein; senescence-associated gene	He and Gan, 2002	*Arabidopsis thaliana*	OSM141989	51	2e-05
ORE 9	AF305597	F-box containing protein ORE9	Woo *et al.*, 2001	*Arabidopsis thaliana*	OSM1359	46	8e-04
ORE 9	AF305597	F-box containing protein ORE9	Woo *et al.*, 2001	*Arabidopsis thaliana*	OSM151086	57	5e-07
At2g44290	AC004521	Unknown protein	Rounsley *et al.*, Unpublished	*Arabidopsis thaliana*	OSM143080	47	6e-05
YLS3	AB047806	Yellow leaf specific	Yoshida *et al.*, 2001	*Arabidopsis thaliana*	OSM1366	95	3e-19

RCC	AJ243066	Red chlorophyll catabolite reductase	Wutrich *et al.*, 2000	*Hordeum vulgare*	OSM128710	309	1e-83
At1g19670	NM_101823	contains similarity to chlorophyllase	Town *et al.*, Unpublished	*Arabidopsis thaliana*	OSM1282	92	4e-18

Table 4.7. RT-PCR analysis of the rice BAC clones in various parts of the rice plant.

Rice BAC clone	Expected size of the transcript (kb)	Expression of the rice BAC clones in various parts of the rice plant							
		Pre-senescing leaf	Senescing leaf	Senesced leaf	Roots	Stems	Leaf sheaths	Panicle with flowers	Panicle with mature grains
OSM146118	0.49	+	+	+	ND	ND	ND	ND	ND
OSM146316	1.75	+	+	+	ND	ND	ND	ND	ND
OSM13394	0.22	+	+	+	+	+	+	+	+
OSM14989	0.60	+	+	+	+	+	+	+	+
OSM151086	0.30	+	+	+	-	-	+	-	-
OSM1282	0.34	+	+	+	+	+	+	+	+
OSM1359	0.12	+	+	+	+	+	+	+	+
OSM1366	0.25	+	+	+	+	-	+	+	+
OSM128710	0.55	+	+	+	+	+	+	+	+

ND = not determined

Chlorophyllase from *Arabidopsis thaliana* had some homology with OSM1282 rice BAC clone (Table 4.6). RT-PCR analysis of the OSM1282 clone, labelled as *chlorophyllase* gene in Figure 4.7C gave 0.14 kb transcript in all the plant parts, a strong 0.35 kb band in the senescing leaf, stem, panicle with flowers and panicle with mature grains but a faint 0.35 kb band in the pre-senescing leaf, senesced leaf and roots (Table 4.7). The RT-PCR product, 0.35 kb transcript, of the senescing leaf has been cloned into pGEM-T Easy vector, sequenced and labelled as C clone. Since the chlorophyllase gene was expressed more in the senescing leaf than in the pre-senescing and senesced leaves, the 0.339 kb transcript was cloned into pGEM-T Easy vector and sequenced. The blastn search result of this C clone showed that it has 100% (339/339) homology with the *Oryza sativa* (japonica cultivar-group) genomic DNA, chromosome 2, BAC clone:OJ1115_D03, working draft sequence. Blastx result of the C clone showed that it has:

a. 40% (49/120) homology with an expressed protein from *Arabidopsis thaliana (GenBank accession number NP 564094).* The gene has GenBank Accession number NM_101823. (Town *et al,* unpublished)
b. 48% (50/103) homology with the AtCHL2 from *Arabidopsis thaliana (GenBank accession number NP_199199* (Town *et al,* unpublished)
c. 37% (43/115) homology with the chlorophyllase from *Citrus unsiu and Citrus sinensis (GenBank accession numbers BAB47176 (*Azuma *et al.,* 2001)*and* AF160869 (Jacob-Wilk *et al.,* 1999)
d. 37% (42/112) homology with the chlorophyllase from *Chenopodium album (GenBank accession number AF134301* (Tsuchiya *et al.,* 1999)

4.4 Discussion

The expression of the *SAG12* gene and the senescence-related genes in rice was investigated. The *SAG 12* gene of *A. thaliana* encodes a cysteine protease which is involved in leaf senescence. The Tblastn search revealed that the SAG 12 protein was homologous to the cysteine protease found in a number of cereals such as rice, barley and *Zea mays*. A phylogenetic analysis of the *SAG12* gene gave 9 groups but the *SAG 12* gene, from *Arabidopsis thaliana*, clustered closest to OSM146118 and OSM146316 rice BAC clones. The OSM146118 rice clone gave the same size and intensity of transcript and was expressed in the same level in the pre-senescing, senescing and senesced leaves which indicate that it was not senescence-specific. However, OSM146316 rice clone gave different size of transcripts, such as 0.18 kb for the pre-senescing leaf, 0.334 kb for the senescing leaf and 0.339 kb for the senesced leaf, which were cloned.

A blastx search of the pre-senescing P1 clone showed that it was homologous to 60K inner-membrane protein homolog of *Treponema pallidum* but the senesced SD3 and SD4 clones showed that they were homologous to an unknown rice protein. A blastx search of the four senescing SG8, SG9, SG10, SG11 and SG12 clones showed high homology with a gene that is similar *Prunus armeniaca's* ethylene-forming-enzyme-dioxygenase. A blastx search of the SG8 clone showed that it was homologous to OSM13394 rice BAC clone. The OSM13394 rice clone showed the presence of a strong and equal intensity of 0.4 kb and 0.25 kb transcripts, in all parts of the rice plant except the stem which indicate that the SG8 clone was not senescence-specific. Hence, efforts were made to isolate senescence-related genes in rice.

The expression of senescence-associated and senescence related genes, such as the *SAG 101, ORE9, red chlorophyll catabolite (RCC) and chlorophyllase* genes, were investigated in rice to identify possible rice promoters that could replace *Arabidopsis* SAG12. These genes had some homology with the rice BAC clones.

SAG101 gene, encoding an acyl hydrolase, is expressed in early-stage senescing leaves (showing up to 25% yellowing) and in late-stage senescing leaves (showing more than 50% yellowing) (He *et al.*, 2002). *SAG101* gene had some homology with OSM14989 rice BAC clone. The OSM14989 clone was expressed in all the plant parts, with highest intensity in the senescing leaf, senesced leaf, stem and leaf sheath and high intensity in the pre-senescing leaf, panicle with flowers and panicle with mature grains. *ORE9* is an F-box protein that regulates leaf senescence in *Arabidopsis* by removing target proteins which are required to delay leaf senescence, hence, it functions to limit leaf longevity (Woo *et al.*, 2001). *ORE9* gene had some homology with OSM1359 and OSM151086 rice BAC clones. The OSM 1359 clone was expressed in equal enormous intensity in all the plant parts, hence, it is not senescence-specific. The OSM151086 clone was expressed in the pre-senescing leaf, senescing leaf, senesced leaf and leaf sheath, hence, it is not senescence-specific. *YLS 3* gene is a yellow-leaf specific gene and its transcript is accumulated at the highest level at an early senescence stage in Arabidopsis thaliana (Yoshida *et al.*, 2001). *YLS 3* gene had some homology with OSM1366 rice BAC clone. The OSM1366 clone was expressed in all plant parts except the roots, hence, it is not senescence-specific. Red chlorophyll catabolite (RCC) reductase catalyzes a key reaction in chlorophyll catabolism (Buchanan, 2000). *RCC* gene from barley, had some homology with OSM128710 rice BAC clone, was expressed in equal enormous intensity in all the plant parts which suggest that it is not senescence-specific. Chlorophyllase is another key enzyme, which degrades the chlorophyll a to chlorophyllide a, in the chlorophyll catabolism (Buchanan, 2000). Chlorophyllase from *Arabidopsis thaliana* had some homology with OSM1282 rice BAC clone. The OSM1282 clone was expressed strongly in the senescing leaf, stem, panicle with flowers and panicle with mature grains but expressed weakly in the pre-senescing leaf, senesced leaf and roots, hence, this clone might be a good candidate for obtaining a senescence-specific promoter. The 0.35 kb transcript, obtained in the senescing leaf after RT-PCR analysis of the OSM1282 clone, has been cloned into pGEM-T Easy vector and labelled as a C clone. Blastn search result of this C clone showed that it has 100% (339/339) homology with the *Oryza sativa* BAC clone. Blastx

result of the C clone showed that it has 40% (49/120) homology with an expressed protein from *Arabidopsis thaliana* (GenBank accession number NP 564094). The gene has GenBank accession number NM_101823; 48% (50/103) homology with the AtCHL2 from *Arabidopsis thaliana* (GenBank accession number NP_199199); 37% (43/115) homology with the chlorophyllase from *Citrus unsiu* and *Citrus sinensis* (GenBank accession numbers BAB47176 and AF160869, respectively); 37% (42/112) homology with the chlorophyllase from *Chenopodium album* (GenBank accession number AF134301).

The expression pattern of *Arabidopsis SAG12* gene and the senescence-related genes showed that they were not senescence-specific in rice. In contrast, Lee *et al.*, (2001) found several clones which are senescence-specific in rice plants. Further work using other strategies or methods is required to determine the expression of other genes and find senescence-specific promoters in rice.

Chapter 5. GENERAL DISCUSSION AND SUMMARY

This research project addressed the following objectives:

1. To determine the changes in chlorophyll content in the top three leaves of field-grown NPT lines during the grain filling period. (Chapter 2)
2. To determine the rate of senescence in the top three leaves of NPT lines and its relationship with grain filling percentage and grain yield. (Chapter 2)
3. To determine the changes in cytokinin levels in the top three leaves of NPT lines and its relationship with the chlorophyll content. (Chapter 2)
4. To transform NPT rice with SAG12-*gus* and SAG12-*ipt* gene using biolistic transformation. (Chapter 3)
5. To determine the integration, expression and inheritance of the *gus* and *ipt* genes in the transgenic SAG12-*gus* and SAG12-*ipt* plants. (Chapter 3)
6. To determine the grain filling percentage and the levels of cytokinins in the leaves of the transgenic SAG12-*ipt* plants. (Chapter 3)
7. To isolate the homologue of the *SAG12* promoter in NPT rice. (Chapter 4)

The success in achieving these objectives is summarised below:

Objective 1. To determine the changes in chlorophyll content in the top three leaves of field-grown NPT lines during the grain filling period.

Poor grain filling is a major problem in many New Plant Type (NPT) lines which may be due to early leaf senescence (Ladha *et al.*, 1998). The top three leaves are the major suppliers of photoassimilates to the developing grains of rice (Yoshida, 1971). Hence, rapid senescence of these leaves might affect grain filling. It is interesting to investigate the difference in the rate of leaf senescence between NPT lines with high grain filling percentage and NPT lines with low grain filling percentage. But before the rate of senescence in these NPT lines can be

determined, it is important to determine the chlorophyll content in the leaves throughout grain filling.

In the present study, the changes in chlorophyll content in the top three leaves of four field-grown NPT lines, with high and low grain filling percentages, were determined. The chlorophyll content in the top three leaves of all the NPT lines declined during grain filling. The chlorophyll decreased earliest in the third leaf followed by the second leaf and then the third leaf which indicate a sequential monocarpic leaf senescence. Previous studies in other rice cultivars have also shown a decline in the chlorophyll content in the top three leaves during grain filling (Mondal and Choudhuri, 1984; Mae *et al.*, 1985; Park *et al.*, 1993; Soejima *et al.*, 1995) and a sequential pattern of leaf senescence (Mondal and Chouduri, 1984). The fast-senescing NPT lines have higher grain filling percentage compared to the slow-senescing NPT lines which indicate that there might be some problem with the transport of assimilates from the elaves to the grains. Yang et al., (2002) also reported that the poor grain filling in japonica/indica hybrid rice was not due to a limitation in source but due poor transport of assimilates to the grains.

Objective 2. To determine the rate of senescence in the top three leaves of NPT lines and its relationship with grain filling percentage and yield

NPT rice promises to raise the current yield ceiling because its capacity to produce 200-250 spikelets per panicle (Peng *et al.*, 1994). However, NPT rice has a problem with poor grain filling (Peng *et al.*, 1999). Ladha *et al.*, (1998) suggested that early leaf senescence may have caused poor grain filling percentage in many NPT lines. In the present study, the difference in the rate of leaf senescence between NPT lines with high grain filling percentage and NPT lines with low grain filling percentage was determined. The fast-senescing NPT lines have higher grain filling percentage compared to slow-senescing NPT lines. Statistical analysis showed that the rate of senescence in the top three leaves of NPT lines was positively and significantly correlated with grain filling percentage.

These results indicate that faster leaf senescence resulted to a higher grain filling percentage. Park *et al.*, (1993) also observed that the rice cultivars with rapid senescence gave a higher grain filling percentage compared to the rice cultivars with slow senescence. Yang *et al.*, (2002) also showed that enhanced leaf senescence, brought about by removing the water irrigation in rice plants after flowering, increased the grain filling percentage. These results imply that translocation or remobilization of nutrients may be a limiting factor in tropical regions (Park, 1993).

Objective 3. To determine the changes in cytokinin levels in the top three leaves of the NPT lines and its relationship with the chlorophyll content

Cytokinins (CKs) are plant hormones which delay leaf senescence and decrease in amount as the leaves reach maturity and senescence. Different methods have been used to extract CKs from the rice leaves. Takagi *et al.*, (1985) used selected ion monitoring and deuterium labelled standards and found only five different kinds of CKs in rice leaves. Saha *et al.*, (1986) found only two different kinds of CKs in the upper four leaves of rice But in this study, 22 different kinds of CKs in the top three leaves were reported for the first time in rice. These 22 CKS were placed into four functionally different groups of CKs, such as, active CKs, storage CKs, inactive CKS, and *cis*-derivatives of zeatin. Among these four groups of CKs, the active CKs are most important because they are involved in delaying leaf senescence.

In previous work, Z and ZR were the active CKs observed in the leaves of rice (Oritano and Yoshida, 1973). The levels of Z rapidly fall with the decrease in chlorophyll content but the ZR level did not change. Yue *et al.*, (1996) reported that the ZR in the flag leaf declined rapidly after flowering in wheat. But Saha *et al.*, (1986) reported that a higher Z and ZR like activity was maintained in the flag leaf than in the other leaves of rice. In this study, however, Z, ZR, DHZ, DHZR, iP and iPR were the active CKs found in the leaves of NPT rice. The level of active CKs in the top three leaves between the fast-senescing NPT line and the

slow-senescing NPT line was compared. Interestingly, the level of active CKs in the flag leaf was two-fold higher in the fast-senescing NPT line than in the slow-senescing NPT line. However, the level of active CKs sharply declined at 2 WAF in the fast-senescing NPT line. This result indicates that it is not the actual content of CKs but the changes in CK dynamics which is the deciding factor for fast senescence. As the active CKs progressively increased, the inactive CKs gradually increased in the leaves of the slow-senescing NPT line. These results indicate that there is a higher rate of biosynthesis/import of active CKs and their conversion to inactive CKs which may be favourable for the delay of leaf senescence.

Objective 4. To transform NPT rice with SAG12-*gus* and SAG12-*ipt* gene using biolistic transformation. (Chapter 3)

Genetic manipulation of cytokinin levels using the *isopentenyl transferase (ipt)* gene fused to the *SAG12* promoter, known as SAG12-*ipt* gene, have delayed leaf senescence and increased grain filling in Minghua, Millin and Misuri rice cultivars (Cao *et al.*, 1999; Lin *et al.*, 2002). Since poor grain filling is a major problem in NPT rice, genetic manipulation of cytokinin levels using the *ipt* gene may improve the grain filling in NPT rice. The SAG12-*ipt* gene was already successfully introduced into tobacco (Gan and Amasino, 1995; Jordi *et al.*, 2000), brocccoli (Chen *et al.*, 2001), lettuce (McCabe *et al.*, 2001) and rice cultivars, namely, Millin, Minghua and Misuri (Cao *et al.*, 1999; Lin *et al.*, 2002) using *Agrobacterium*-mediated transformation and in wheat (Daskalova *et al.*, 2002) using biolistic transformation. In this study, the SAG12-*ipt* gene was successfully introduced into NPT rice using biolistic transformation.

Objective 5. To determine the integration, expression and inheritance of the *gusA* and *ipt* genes in the transgenic SAG12-*gusA* and SAG12-*ipt* plants. (Chapter 3)

In the present study, the ipt gene was present in 221 prmiary transformants. An intact copy of the SAG12-*ipt* gene was successfully integrated in the rice genome of a number of these transgenic T_0 plants. A high copy number and multiple

rearrangements of the SAG12-*ipt* gene were observed in the transgenic T_0 plants. The *ipt* gene was expressed in the leaves of the transgenic T_0 plants. The *ipt* gene was inherited in the T_1 progeny of thetransgenic T_0 plants. A 3:1 segregation ratio was observed in the T_1 progeny of line T1193 which indicates that the *ipt* gene was inherited as a single Mendelian locus. Four T_1 progeny of line T1193 and their T_2 progeny showed delayed leaf senescence, shorter plant height, two to four week delay in flowering and poor grain filling percentage

Objective 6. To determine the grain filling percentage and the levels of cytokinins in the leaves of the transgenic SAG12-*ipt* plants. (Chapter 3)

The levels of different cytokinins in the leaves of transgenic lines T1193 and T2068 increased in various amounts. The levels of Z, Z9R, iP and iP9R in the leaves of transgenic line T2068 and the levels of Z9R, DHZ9R and iP in the leaves of transgenic line T1193 were higher than in the control plants. Cytokinin analysis of the T_2 progeny of line T1193 showed that the levels of Z9R and iP in the leaves of T1193-24-9 plant and the levels of Z9R, DHZ9R, iP and iP9R in the leaves of T1193-27-8 plant were higher than in the control plants. This cytokinin increase might have affected some aspects in plant development, such as plant height and delayed flowering, in the transgenic SAG12-*ipt* plants.

Delayed leaf senescence was observed in the leaves of SAG12-*ipt* lettuce (McCabe *et al.*, 2001), SAG12-*ipt* broccoli (Page *et al.*, 2001) and SAG12-*ipt* rice (Cao *et al.*, 1999; Lin *et al.*, 2002). A two-week delay in flowering and a high degree of sterility were reported in transgenic SAG12-*ipt* wheat and tobacco. A shorter plant height and delayed flowering were reported in transgenic tobacco plants containing the *ipt* gene (Hewelt *et al.*, 1994). The transgenic plants containing the *ipt* gene have delayed leaf senescence and increased levels of cytokinins (Smart *et al.*, 1991; Gan and Amasino, 1995; Smigocki, 1995; Jordi *et al.*, 2000).

Endogenous manipulations of cytokinin content even only relatively modest increases in cytokinin levels result in major developmental changes (Hewelt *et al.*, 1994). The expression of the *ipt* gene resulted to delayed leaf senescence and increased seed yield without any changes in the plant morphology.in tobacco (Gan and Amasino, 1995) and in rice (Cao, 2001; Lin *et al.*, 2002) but with a two week-delay in flowering of wheat (Daskalova *et al.*, 2002). Most of the previous findings reported that the expression of *ipt* gene, under the control of different types of promoters, also resulted to morphological changes in other crops (Hewelt *et al.*, 1994; Smigocki, 1995, McKenzie *et al.*, 1998; Medford *et al.*, 1989; Smart *et al.*, 1991; Chen *et al.*, 2001; Gapper *et al.*, 2002; McKenzie *et al.*, 1998; McCabe *et al.*, 2001). Our study has shown that expression of the *ipt* gene produced cytokinins which delayed in leaf senescence, delayed flowering by four weeks, shorter plant height and poor grainfilling. As shown in the previous study, delayed senescence in two field-grown NPT lines resulted to lower percentage of grainfilling which might be due to the assimilates were not effectively transported to the grains.

Objective 7: To isolate the rice homologue of the *SAG12* promoter.

The expression of *Arabidopsis SAG12* gene and senescence-related genes was investigated in NPT rice to identify possible rice promoters that could replace the *Arabidopsis SAG12* promoter. A phylogenetic tree showed that the *Arabidopsis SAG12* gene clustered closest to OSM146118 and OSM146316 rice BAC clones. However, the expression of these rice BAC clones in different types of leaves indicate that they were not senescence-specific in rice. The senescence related genes, such as *SAG 101, ORE9, red chlorophyll catabolite, YLS3 and chlorophyllase* genes, had some homology with OSM14989, OSM1359 and OSM151086, OSM1366, OSM128710 and OSM1282 rice BAC clones, respectively. However, these rice BAC clones were expressed in all or most parts of the rice plant which indicate that they were not senescence-specific. However, the OSM1282 rice BAC clone was expressed strongly in the senescing leaf, stems, panicle with flowers and panicle with mature grains but expressed weakly in the

pre-senescing leaf, senesced leaf and roots. These results indicate that this rice clone might be a good candidate for obtaining a senescence-specific promoter. The 0.35 kb transcript obtained in the senescing leaf has been cloned. The blastn search result of this clone showed that it has 100% homology with the *Oryza sativa* but blastx result showed that it has 37% homology with the chlorophyllase from *Citrus unsiu* and *Citrus sinensis.*

FUTURE WORK:

The next direction worth pursuing is to fuse the *ipt* gene with the endosperm-specific promoters (SBE and Gt1) which drive gene expression abundantly and specifically in developing seeds with a maximum in the middle and late-middle stages of endosperm development. These SBE-*ipt* and Gt1-*ipt* gene contructs should be introduced into NPT rice. The cytokinin levels in the developing grains will be measured during the grain filling period. The grain filling percentage of the transgenic plants will be assesed.

Another direction is to utilize other approaches/methods of isolating the rice homologue of the SAG12 promoter. Subtractive hybridization, differential display or screening a cDNA library of different types of rice leaves can be utilized in finding the rice senescence specific promoter or rice-equivalent of the SAG12 promoter. The isolated rice promoter can be fused to the *ipt* gene and introduced into NPT rice. The transgenic plants produced will be evaluated at the molecular and physiological level.

Chapter 6. References

Ainley WM, McNeil KJ, Hill JW, Lingle WL, Simpson RB, Brenner ML, Nagao RT and Key JL (1983) Regulatable endogenous production of cytokinins up to 'toxic' levels in transgenic plants and plant tissues. *Plant Mol Bio* 22:13-24

Akiyoshi, DE, Klee H, Amasino R, Nester EW and Gordon MP (1984). T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. *Proc Natl Acad Sci USA* 81:5994-5998

Aldemita R and Hodges TK (1996) *Agrobacterium tumefaciens*-mediated transformation of elite indica rice varieties. *Planta* 199:612-617

Ambler JR, Morgan PW and Jordan WR (1992) Amounts of zeatin and zeatin riboside in xylem sap of senescent and non-senescent sorghum. *Crop Sci* 32(2):411-419

Aoyama T and Chua NH (1997) A glucocorticoid-mediated transcriptional induction system in transgenic plants. *Plant J* 11:605-612

Azuma RK, Kurata H, Shimokawa K and Adachi M (2001) Chlorophyllase on ethylene-treated *citrus unshiu* Marc Published Only in Database

Battraw MJ and Hall TC (1990) Histochemical analysis of CaMV promoter (β-glucoronidase gene expression in transgenic rice plants. *Plant Mol Bio* 15:527-538

Becker D, Brettschneider R and Lorz H (1994) Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. *The Plant J* 5:299-307

Beinsberger SEI, Valcke RLM, Clijsters HMM, De Greef JA and Van Onckelen H (1992) Effects of enhanced cytokinin levels in *ipt* transgenic tobacco. In: Kaminek M, Mok DWS and Zazimalova E (eds) Physiology and Biochemistry of Cytokinins in Plants. The Hague: SPB Academic Publishing. pp. 77-82

Benfey PN and Chua NH (1990) The Cauliflower Mosaic Virus (CaMV 35S) promoter: combinational regulation of transcription in plants. *Science* 250:959-966

Birch RG and Bower R (1994) Principles of gene transfer using particle bombardment. In: Particle Bombardment Technology for Gene Transfer, Yang NS and Christou P (eds) New York: Oxford University. pp.3-37

Biswal B and Biswal UC (1999) Leaf senescence: physiology and molecular biology. *Current Sci* 77(6):775-782

Borlaug NE (2002) Feeding a world of 10 billion people: the miracle ahead. *In Vitro Cell Dev Biol Plant* 38:221-228

Borrell AK, Hammer GL and Henzell RG (2000) Does maintaining green leaf area in sorghum improve yield under drought? II. Dry matter production and yield. *Crop Sci* 40(4):1037-1048

Bowen BA (1993) Markers for plant gene transfer. In: Transgenic Plants, Kung S and Wu R (ed), San Diego: Academic pp.89-123

Bower R and Birch RG (1992) Transgenic sugarcane plants via microprpojectile bombardment. *Plant J* 2:409-416

Bruce WB, Christensen AH, Klein T, Fromm M and Quail PH (1989) Photoregulation of a phytochrome gene promoter from oat transferred into rice by particle bombardment. *Proc Natl Acad Sci USA* 86:9692-9696.

Buchanan B (2000) Biochemistry and Molecular Biology of Plants. Rockville, MD: American Society of Plant Physiologists. pp. 1367

Buchanan-Wollaston V (1997) The molecular biology of leaf senescence. *J Exp Bot* 48:181-199

Burkhardt P, Beyer P, Wunn J, Kloti A, Armstrong GA, Schledz M, von Lintig J and Potrykus I (1997) Transgenic rice (*Oryza sativa*) endosperm expressing daffodil (*Narcissus pseudonarcissus*) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis. *Plant J* 11(5):1071-1078

Cao J, Duan XL, Mc Elroy D and Wu R (1992) Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. *Plant Cell Rep* 11:586-591

Cao J, Zhang W, McElroy D and Wu R (1991) Assessment of rice genetic transformation techniques. In: Khush GS and Toenniesen G (eds) Rice Biotechnology. International Rice Research Institute. PO Box 933, Manila, Philippines. pp.175-198.

Cao M (2001) Performance of autoregulatory senescence-inhibition gene in rice. *Hunan Agric and Tech Newsl* 2(2):17-23

Casas AM, Kononowicz AK, Zehr UB, Tomes DT, Axtell JD, Butler LG, Bressan RA and Hasegawa PM (1993) Transgenic sorghum plants via microprojectile bombardment. *Proc Natl Acad Sci* USA 90:11212-11216

Castillo AM, Vasil V and Vasil IK (1994) Rapid production of fertile transgenic plants of rye (*Secale cereale* L.). *Bio/Tech* 12:1366-1371

Chalfie M, Tu Y, Euskirchen G, Ward WW and Prasher DC (1994) Green fluorescent protein as a marker for gene expression. *Science* 263:802-805

Chamberlain D, Bretell R, Last D, Witrzens B, Mc Elroy D, Dolferus R and Dennis ES (1994) The use of the Emu promoter with antibiotic and herbicide resistance genes for the selection of transgenic wheat callus and rice plants. *Austr J Plant Physiol* 21: 95-112

Chen L-F, Hwang J-H, Charng Y-Y, Sun C-W and Yang S-F (2001) Transformation of broccoli (*Brassica oleracea* var. italica) with *isopentenyl transferase* gene via

Agrobacterium-tumefaciens for pos-harvest yellowing retardation. *Molec Breed* 7:243-257

Chen S, Lin HX, Xu G and Zhang Q (2000) Improvement of bacterial resistance of 'Minghui 63,' an elite restorer line of hybrid rice, by molecular marker-assisted selection. *Crop Sci* 40:239-244.

Chen L, Marmey P, Taylor NJ, Brizard JP, Espinoza C, deCruz P, Huet H, Zhang SP, de Kocko A, Beachy RN and Fauquet CM (1998) Expression and inheritance of multiple transgenes in rice plants. *Nat Biotech* 16:1060-1064

Cheng XR, Sardana R, Kaplan H and Altosaar I (1998) *Agrobacterium*-transformed rice plants expressing synthetic *cryIA(b)* and *cryIA(c)* genes are highly toxic to striped stem borer and yellow stem borer. *Proc Natl Acad Sci USA* 95:2767-2772

Christensen AH, Sharrock RA and Quail PH (1992) Maize polyubiquitin genes – structure, thermal perturbation of expression and transcript splicing and promoter activity following transfer to protoplasts by electroporation. *Plant Mol Bio* 18:675-689

Christou P, McCabe DE and Swain WF (1988) Stable transformation of soybean callus by DNA-coated gold particles. *Plant Physiol* 87:671-674

Christou P, Ford T and Kofron M (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. *Bio/Tech* 8:736-740

Christou P (1992) Genetic transformation of crop plants using microprojectile bombardment. *The Plant J* 2:275-281

Christou P and Ford T (1995) The impact of selection parameters on the phenotype and genotype of transgenic rice callus and plants. *Transgenic Res* 4:44-51

Christou P (1997) Rice transformation: bombardment. *Plant Mol Biol* 35:197-203

Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY and Bi FY (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. *Scientia Sinica* 5:659-668

Cornejo MJ, Luth D, Blankenship KM, Anderson OF and Blech AE (1993) Activity of maize ubiquitin promoter in transgenic rice. *Plant Mol Bio* 23:567-581

Daskalova S, Sorokin A, Scott NW, Slater A, Fowler MR, Kaminek M, Gaudinova A, Trckova M and Elliott MC (2002) Enhancement of wheat grain yields by delaying leaf senescence. 10th International Plant Tissue Culture and Biotechnology. Florida

Datta K, Torrizo L, Oliva N, Alam MF, Wu C, Abrigo E, Vasquez A, Tu J, Quimio C, Alejar M, Nicola Z, Khush GS and Datta SK (1996) Production of transgenic rice by protoplast, biolistic and *Agrobacterium* systems. *Proc Vth Internat Symp Rice Mol Biol* pp. 159-167

Datta K, Oliva N, Torrizo L, Alam MF, Oliva N, Abrigo E, Khush GS and Datta SK (1998) Constitutive and tissue-specific differential expression of *cryIA(b)* gene in transgenic rice plants conferring enhanced resistance to rice insect pests. *Theor Appl Genet* 97:20-30.

Datta K, Baisakh N, Thet KM, Tu J and Datta SK (2002) Pyramiding transgenes for multiple protection in rice against bacterial blight, stem borer and sheath blight. *Theor Appl Genet* 106: 1-8.

Datta K, Baisakh N, Oliva N, Torrizo L, Abrigo E, Tan J, Rai M, Rehana S, Al-Babili S, Beyer P, Potrykus I and Datta SK (2003) Bioengineered 'golden' indica rice cultivars with β-carotene metabolism in the endosperm with hygromycin and mannose selection systems. *Plant Biotech J* 1:81-90

Datta K, Tu J, Oliva N, Ona I, Velazhahan R, Mew TW, Muthukrishnan S and Datta SK (2001) Enhanced resistance to sheath blight by constitutive expression of

infection-related rice chitinase in transgenic elite indica rice cultivars. *Plant Sci* 160:405-414

Datta SK, Peterhans, A, Datta K and Potrykus I (1990b) Genetically engineered fertile indica-rice recovered from protoplasts. *Bio/Tech* 8:736-740

Datta, S, Peterhans A, Datta K, and Potrykus I (1990a) A polyethylene glycol-mediated protoplast transformation system for production of fertile transgenic rice plants. *Plant Physiol* 93:857-863

Datta SK, Datta K, Soltanifar N, Donn G and Potrykus I (1992) Herbicide-resistant Indica rice plants from IRRI breeding line IR72 after PEG-mediated transformation of protoplast. *Plant Mol Bio* 20:619-629

Datta S (1999) Transgenic cereals: *Oryza sativa* (rice). In: Molecular Improvement of Cereal Crops. Vasil IK (ed), The Netherlands: Kluwer Academic Publishers. pp.149-187

Davey M, Ingram H, Azhakanandam K and Power JB (2000) The genetic transformation of rice and maize. In: Cereal Biotechnology. Morris PC and Bryce JH (eds), Cambridge: Woodhead Publishing Limited.pp.43-69

Dellaporta SL, Wood VP and Hicks JB (1983) A plant DNA mini-preparation: version II. *Plant Mol Biol Reptr* 1: 19-21

Dekeyser R, Claes B, Marichal M, Van Montagu M and Caplan A (1989) Evaluation of selectable markers for rice transformation. *Plant Physiol* 90:217-223

Dobrev P and Kaminek M (2002) Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction. *J Chrom* 950:21-29

Duan X, Li X, Xue Q, Abo-El-saad M, Xu D and Wu R (1996) Transgenic rice plants harboring an introduced potato *proteinase inhibitor II* gene are insect resistant. *Nat Biotech* 14:494-498

Estruch JJ, Prinsen E, Van Onckelen H, Schell J and Spena A (1991) Viviparous leaves produced by somatic activation of an inactive cytokinin-synthesizing gene. *Science* 254:1364-1367

Estruch JJ, Granell A, Hansen G, Prinsen E, Redig P, Van Onckelen H, Schwarz-Sommer Z, Sommer HJ and Spena A (1993) Floral development and expression of floral homeotic genes are influenced by cytokinins. *Plant J* 4:379-384

Faiss M, Zalubilova J,Strnad M and Schumulling T (1997) Conditional transgenic expression of the *ipt* gene indicates a function for cytokinins in paracrine signalling in whole tobacco plants. *Plant J* 12:401-415

FAOSTAT (2003) Statistical Databases. Food and Agriculture Organization (FAO) of the United Nations, Rome. http://www.fao.org

Finch RP (1994) An introduction to molecular biotechnology. In: Molecular Biology in Crop Protection. Marshall G and Walters D (eds), London: Chapman and Hall. pp 1-37

Finer JJ, Vain P, Jones MW and McMullen MD (1992) Development of the particle inflow gun for DNA delivery to plant cells. *Plant Cell Rep* 11:323-328

Finnegan J and McElroy D (1994) Transgene inactivation: plants fight back. *Bio/Tech* 12:883-888

Flavell RB (1994). Inactivation of gene expression inplants as a consequence of specific sequence duplication. *Proc Natl Acad Sci USA* 91:3490-3496

Franks T and Birch RG (1991) Microprojectile techniques for direct gene transfer into plant cells. In: Advanced Methods in plant breeding and Biotechnology. Murray DR (ed), Wallingford, England: CAB International pp.103-127

Fromm ME, Morrish F, Armstrong C, Williams R, Thomas J and Klein TM (1990) Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. *Biotech* 8:833-839

Fu X, Duc LT, Fontana S, Bong BB, Tingjuangjun P, Sudhakar D, Twyman RM, Christou P and Kohli A (2000) Linear transgenic constructs lacking vector backbone sequences generate low-copy number transgenic plants with simple integration patterns. *Transgenic Res* 9:11-19

Fujimoto H, Itoh K, Yamamoto M, Yukuza J and Shimamoto K (1993) Insect resistant rice generated by introduction of a modified d-endotoxin gene *of Bacillus thuringensis*. *Bio/Tech* 11:1151-1155

Gan S and Amasino R (1997) Making sense of senescence. Molecular genetic regulation and manipulation of leaf senescence. *Plant Physiol* 113:313-319

Gan S and Amasino RM (1995) Inhibition of leaf senescence by autoregulated production of cytokinin. *Science* 270(5244): 1986-1988

Gan S and Amasino R M (1996) Cytokinins in plant senescence: from spray and pray to clone and play. *BioEssays* 18: 557-565.

Gapper NE, McKenzie MJ, Christey MC, Braun RH, Coupe SA, Lill RE and Jameson PE (2002) *Agrobacterium*-mediated transformation to alter ethylene and cytokinin biosynthesis in broccoli. *Plant Cell, Tiss and Org Cult* 70:41-50.

Gardner E, Simmons M and Nustad DP (1991) Principles of Genetics. 8th ed. New York: John Wiley and Sons, Inc. pp. 649

Garnier F, Carpin S, Label P, Creche J, Rideau M and Hamdi S (1996) Effect of cytokinin on alkaloid accumulation in periwinkle callus cultures transformed with a light-inducible *ipt* gene. *Plant Sci* 120:47-55

Garrison FR, Brinker AM and Nooden LD (1984) Relative activities of xylem-supplied cytokinins in retarding soybean leaf senescence and sustaining pod development. *Plant and Cell Physiol* 25(2):213-224

Gelvin, SB (2000) *Agrobacterium* and plant genes involved in T-DNA transfer and integration. *Ann Rev Plant Physiol Plant Mol Bio* 51:223-256

Ghareyazie B (1995) Transformation of indica and other rices (*Oryza sativa* L.): Genetic integration, expression, inheritance and enhanced insect resistance. PhD thesis, UPLB. pp.217.

Ghareyazie B, Menguito CA, Rubia LG, De Palma, JM, Liwanag EA, Alinia AF, Cohen M, Muthukhrishnan S, Khush G and Bennett J (1997) Enhanced resistance to two stem borers in an aromatic rice containing a synthetic *cryIA(b)* gene. *Molec Breed* 3:401-414

Ghosh Biswas GC, Chen D-F and Elliott MC (1998) A routine system for generation of transgenic rice (*Oryza sativa* L.) plants by microprojectile bombardment of embryogenic cell clusters. *Plant Sci* 133:203-210

Godwin, I (2003) Plant germplasm collections as sources of useful genes. In: Plant Molecular Breeding. Newbury HJ (ed), Blackwell Publishing Limited. Oxford. pp.134-151

Gonzalez A, Jimenez A, Vasquez D, Davies J and Schindler D (1978) Studies on the mode of action of hygromycin B, an inhibitor of translocation in eukaryotes. *Biochimica et Biophysica Acta* 521:459-469

Gordon-Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WG, O'Brien JV, Chambers SA, Adams WR, Willets NG, Rice TB, Mackey CJ, Krueger

RW, Kausch AP and Lemaux PG (1990) Transformation of maize cells and regeneration of fertile transgenic plants. *The Plant Cell* 2(7):603-618

Gritz L and Davies J (1983) Plasmid-encoded hygromycin B resistance: the sequence of *hygromycin phosphotranferase* gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. *Gene* 179-188

Groot SPC, Bouwer R, Busscher M, Lindhout P and Dons HJ (1995) Increase of endogenous zeatin riboside by introduction of the *ipt* gene in wild type and the lateral suppressor mutant of tomato. *Plant Growth Regul* 16:27-36

Guerineau F (1995) Tools for expressing foreign genes in plants. In: Methods in Molecular Biology. Vol.49: Plant Gene Transfer and Expression Protocols. Tuan R (ed), New Jersey: Humana Press Inc Totowa pp.1-32

Gura T (1999) New genes boost rice nutrients. *Science* 285:994-995

Haberer G and Kieber JJ (2002) Cytokinins - new insights into a classic phytohormone. *Plant Physiol* 128:354-362

Hagio T, Blowers AD and Earle ED (1991) Stable transformation of sorghum cell cultures after bombardment with DNA-coated microprojectiles. *Plant Cell Rep* 10:260-264

Hamdi S, Creche J, Garnier F, Mars M, Decendit A, Haspar T and Rideau (1995) Cytokinin involvement in the control of coumarin accumulation in *Nicotiana tabacum*. Investigations with normal and transformed tissues carrying the *isopanetenyl transferase* gene. *Plant Physiol Biochem* 33(3):283-288

Hayakawa T, Zhu TY, Itoh K, Kimura Y, Izawa T, Shimamoto K and Toriyama S (1992) Genetically engineered rice resistant to rice stripe virus, an insect-transmitted virus. *Proc Natl Acad Sci USA* 89:9865-9869

Han S, Wu Z, Yang H, Wang R, Yie Y, Xie L and Tien P (2000) Ribozyme-mediated resistance to rice dwarf virus and the transgenic silencing in the progeny of transgenic rice plants. *Trangen Res* 9:195-203

Hayashimoto A, Li Z and Murai N (1990) A polyethylene glycol-mediated protoplast transformation system for production of fertile transgenic rice plants. *Plant Physiol* 93:857-863

He Y and Gan S (2002) A gene encoding an acyl hydrolase is involved in leaf senescence in *Arabidopsis*. *Plant Cell* 14(4): 805-815.

Herrera-Estrella L, Leon P, Olsso O and Teeri TH (1983) Reporter genes for plants. In: Plant Moleculat Biology Manual, Gelvin SB and Schilperoort RS (eds) Dordrect: Kluwer Academic Publishers. pp.1-32

Hewelt A, Prinsen E, Schell J, Van Onkelen H and Schumulling T (1994) Promoter tagging with a promoterless *ipt* gene leads to cytokinin-induced phenotypic variability in transgenic tobacco plants: implications of gene dosage effects. *Plant J* 6:879-891

Hiei Y, Komari T and Kubo T (1997) Transformation of rice mediated by *Agrobacterium tumefaciens*. *Plant Mol Bio* 35:205-218

Hiei Y, Ohta S, Komari S and Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J* 6:271-282

Ho SL, Tong WF and Yu SM (2000) Multiple mode regulation of a cysteine proteinase gene expression in rice. *Plant Physiol* 122 (1): 57-66

Hoshida H, Hibino T, Hayashi Y, Tanaka A, Takabe T and Takabe (2000) Enhanced tolerance to salt stress in transgenic rice that overexpresses chloroplast glutamine synthetase. *Plant Mol Biol* 43:103-111

Hu C and Wang P (1986) Embryo culture: technique and application. In: Handbook of Plant Cell Culture. Vol 4. Evans DA, Sharp W and Ammirato PB (eds), Macmillan, New York, pp.43-96

Jacob-Wilk D, Holland D, Goldschmidt EE, Riov J and Eyal Y (1999) Chlorophyll breakdown by chlorophyllase: isolation and functional expression of the *chlase1* gene from ethylene-treated *Citrus* fruit and its regulation during development. *Plant J* 20 (6): 653-661

Jahne A, Becker D, Brettschneider R and Lorz H (1994) Regeneration of transgenic, microspore-derived fertile barley. *Theor Appl Genet* 89:525-533

Jain RK, Jain S, Wang B and Wu R (1996) Optimization of biolistic method for transient gene expression and production of agronomically useful transgenic Basmati rice plants. *Plant Cell Rep* 15:963-968

Jefferson RA (1987) Assaying chimeric genes in plants: the *gus* gene fusion system. *Plant Mol Biol Rep* 5:387-405

Jefferson RA (1989) The GUS reporter gene system. *Nature* 342(6251):837-838

Jena KK and Khush GS (1984) Embrvo rescue of interspecific hybrids and its scope in rice improvement. *Rice Genet Newsl* 1: 133-134.

Joersbo M, Donaldson I, Kreiberg J, Petersen SG, Brundstedt J and Okkels FT (1998) Analysis of mannose selection used for transformation of sugarbeet. *Molec Breed* 4:111-117

Jones M (2002) Plant Biotechnology. In: Molecular Biology and Biotechnology. Walker J and Rapley R (eds), The Royal Society of Chemistry, Cambridge, pp. 203-236

Kaminek M, Trckova M, Motyka V, Daskalova S and Elliott MC (2003) Cereal improvement by exploitation of the mechanisms regulating levels *(Plenary Lecture).* In: NATO-Russia Advanced Research Workshop: Phytohormones in "Plant Biotechnology and Agriculture" Moscow, Russia 12-16 May 2002, Machakova I and Romanov GA (eds), pp. 109-128

Kato H, Shintani A and Minamikawa T (1999) The structure and organization of two cysteine endopeptidase genes from rice. *Plant Cell Physiol* 40(4):462-467

Keil M, Sanchez-Serrano JJ and Willmitzer L (1989) Both wound-inducible and tuber specific expression are mediated by promoter of a single member of the potato *proteinase inhibitor II* gene family. *EMBO J* 8:13230-1330

Khush GS (1995) Breaking the yield frontier of rice. *GeoJournal.* 35:329-332

Khush, GS (1997) Origin, dispersal, cultivation of rice. *Plant Molec Biol* 35:25-34

Khush GS and Peng S (1996) Breaking the yield frontier of rice. In: Increasing Yield Potential in Wheat: Breaking the Barriers, Ciudad Obregon, Sonora, 26-28 March 1996. Reynolds M (ed), Mexico: International Maize and Wheat Improvement Center. pp.36-51

Khush GS and Virk PS (2000) Rice breeding: achievements and future strategies. *Crop Improv* 27(2):115-144

Khush GS and Virk PS (2002) Rice improvement: past, present and future. In: Crop Improvement – Challenges in the Twenty First Century. Kang MS (ed), New York: Food Products Press, Inc. pp.17-42.

Kim HS (1993) Protoplast culture and use of PEG and biolistic methods of transformation in rice (*Oryza sativa* L.) A PhD thesis submitted to the University of the Philippines. pp.1-105

Klee HJ (1994) Transgenic plants and cytokinin biology. In: Mok DWS and Mok MC (Eds.) Cytokinins: Chemistry, Activity and Function. Ann Arbor: CRC Press. pp.289-293

Klein TM, Fromm ME, Gradziel T and Sanford JC (1988a) Factors influencing gene delivery into *Zea mays* cells by high-velocity microprojectiles. *Bio/Tech* 6:559-563

Klein TM, Fromm ME, Weissinger A, Tomes D, Schaff S, Sleeten M and Sanford JC (1988b) Transfer of foreign genes into intact maize cells using high velocity microprojectiles. *Proc Natl Acad Sci USA* 85:4305-4309

Klein TM, Harper EC, Svab Z, Sanford JC, Fromm ME and Maliga P (1988c) Stable genetic transformation of intact *Nicotiana* cells by the particle bombardment process. *Proc Natl Acad Sci USA* 85:8502-8505

Koehler SM and Ho TH (1990) Hormonal regulation, processing, and secretion of cysteine proteinases in barley aleurone layers. *Plant Cell* 2 (8), 769-783

Komari T, Hiei Y, Saito Y, Murai N and Kumashiro T (1996) Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. *Plant J* 10:165-174

Koziel MG, Beland GL, Bowman C, Carrozi NB, Crenshaw R, Crossland L, Dawson J, Desai N, Hill M, Kadwell S, Launis K, Lewis K, Maddox D, McPherson K, Meghji MR, Merli E, Rhodes R, Warren GW, Wright M and Evola SV (1993) Field performance of elite maize plants expressing an insecticidal protein derived from *Bacillus thuringensis*. *Bio/Tech* 11:194-200

Krishnamurthy K and Giroux MJ (2001) Expression of wheat puroindoline genes in transgenic rice enhances grain softness. *Nat Biotech* 19:162-166

Ku SB, Agarie S, Nomura H, Fukayama H, Tsuchida H, Ono K, Hirose S, Toki S, Miyao M and Matsuoka M (1999) High-level expression of maize phosphoenolpyruvate carboxylase in transgenic rice plants. *Nat Biotech* 17:76-80

Kuhlemeir C, Green PJ and Chua NH (1987) Regulation of gene expression in higher plants. *Ann Rev Plant Physiol* 38:221-257

Kusaba S, Kano-Murakami Y, Matsuoka M, Tamaoki M, Sakamoto T, Yamaguchi I and Fukumoto M (1998) Alteration of hormone levels in transgenic tobacco plants overexpressing the rice homeobox gene OSH1. *Plant Physiol* 116:471-476

Kyozuka J, Fujimoto H, Izawata T and Shimamoto K (1991) Anaerobic induction and tissue-specific expression of maize Adh1 promoter in transgenic rice plants and their progeny. *Mol Gen Genet* 228:40-48

Kyozuka J, McElroy D, Hayakawa T, Xie Y, Wu R and Shimamoto K (1993) Light regulated and cell-specific expression of tomato *rbcS-gusA* fusion genes in transgenic rice. *Plant Physiol* 102:991-1000

Ladha JK, Kirk GJD, Bennett J, Peng S, Reddy CK, Reddy PM and Singh U (1998) Opportunities for increased nitrogen-use efficiency from improved lowland rice germplasm. *Field Crops Res* 56:41-71

Lee RH, Wang CH, Huang LT and Chen SCG (2001) Leaf senescence inrice plants: cloning and characterization of senescence up-regulated genes. *J Exp Bot* 52: 1117–1121

Li ZJ, Burow MD and Murai N (1991) High frequency generation of fertile transgenic rice plant after PEG-mediated protoplast transformation. *Plant Mol Bio Rep* 8:276-291

Li Y, Hagen G and Guilfoyle TJ (1992a) Altered morphology in transgenic tobacco plants that overproduce cytokinins in specific tissues and organs. *Dev Biol* 153:386-395

Li XQ, Liu CN, Ritchie SW, Peng JY, Gelvin SB and Hodges TK (1992b) Factors influencing *Agrobacterium*-mediated transient expression of *gusA* in rice. *Plant Mol Bio* 20:1037-1048

Li Z, Xie Q, Rush bM and Murai N (1992c) Fertile transgenic rice plants generated via protoplasts from the U.S. Cultivar Labelle. *Crop Sci* 32:810-814

Li L, Qu R, de Kochko A, Fauquet C and Beachy RN (1993) An improved rice transformation system using the biolistic method. *Plant Cell Rep* 12:250-255

Li Z, Upadhyaya M, Meena S, Gibbs A and Waterhouse PM (1997) Comparison of promoters and selectable marker genes for use in Indica rice transformation. *Molec Breed* 3:1-14

Lin W, Anuratha CS, Datta K, Potrykus I, Muthukrishnan S and Datta SK (1995) Genetic engineering of rice for resistance to sheath blight. *Bio/Tech*13:686-691

Lin YJ, Cao M, Xu G, Chen H, Wei J and Zhang Q-F (2002) Cultivating rice with delaying leaf senescence by P_{SAG12}-*IPT* gene transformation. *Acta Bot Sinica* 44(11):1333-1338

Lloyd AM and Davis RW (1994) Functional expression of the yeast FLP/FRT site-specific recombination system in *Nicotiana tabacum*. *Mol Gen Genet* 242:653-657.

Lohman K, Gan S, John M and Amasino RM (1994) Molecular analysis of natural leaf senescence in *Arabidopsis thaliana*. *Physiol Plant* 92:322-328

Lok F and Sorensen MB Barley mRNA encoding cysteine endopeptidase EP-A precursor. Unpublished

Lonsdale D, Onde S and Cuming A (1990) Transient expression of exogenous DNA in intact, viable wheat embryos following particle bombardment. *J Exp Bot* 41:1161-1165

Lucca P, Ye X and Potrykus I (2001) Effective selection and regeneration of transgenic rice plants with mannose as selective agent. *Molec Breed* 7:43-49

Lu X, Zhang Z and Virmani SS hybrid rice (1994) Breeding status quo of two-line system hybrid rice. *Chinese J Rice Sci* 8:48-54

Luthra R, Dubey RK, Srivastava AK and Kumar S (1997) Microprojectile mediated plant transformation: a bibliographic search. *Euphytica* 95:269-294

Maclean JL, Dawe DC, Hardy B and Hettel GP (2002) Rice Almanac 3rd edition Philippines: International Rice Research Institute. pp. 253

Mae T, Hoshino T and Ohira K (1985) Proteinase activities and loss of nitrogen in the senescing leaves of field-grown rice (*Oryza sativa* L.). *Soil Sci Plant Nutr* 31(4):589-600

Maheswaran M, Subudhi PK, Nandi S, Xu JC, Parco A, Yang DC and Huang N (1997) Polymorphism, distribution and segregation of AFLP markers in a double haploid rice population. *Theor Appl Genet* 94:39-45

Maqbool SB and Christou P (1999) Multiple traits of agronomic importance in transgenic indica rice plants: analysis of transgene integration patters, expression levels and stability. *Molec Breed* 5:471-480

Martineau B, Houck CM, Sheehy RE and Hiatt WR (1994) Fruit-specific expression of the *A .tumefaciens isopentenyl transferase* gene in tomato: effects on fruit ripening and defense-related gene expression in leaves. *The Plant J* 5(1):11-19

Matsuoka M, Kyozuka J, Shimamoto K and Kano-Murakami Y (1994) The promoter of two carboxylase in a C4 plant (maize) direct cell-specific, light regulated expression in a C3 plant (rice). *Plant J 6:311-319*

Matsuki R, Onodera H, Yamauchi T and Uchimiya H (1989) Tissue-specific expression of the *rol*C promoter of the Ri plasmid in transgenic rice plants.source

McCabe DE, Swain WF, Martinell BJ and Christou P (1988) Stable transformation of soybean (*Glycine max*) by particle acceleration. *Bio/Tech* 6:923-926

McCabe DE and Martinell BJ (1993) Transformation of elite cotton cultivars via particle bombardment of meristems. *Bio/Tech* 11:596-598.

McCabe MS, Garratt LC, Schepers F, Jordi W, Stoopen GM, Davelaar E, van Rhijn HA, Power JB and Davey MR (2001) Effects of P_{SAG12}-*IPT* gene expression on development and senescence in transgenic lettuce. *Plant Physiol* 127:505-516

McCouch SR, Kochert G, Yu ZH, Wang ZY, Khush GS, Coffman WR and Tanksley SD (1988) Molecular mapping on rice chromosomes. *Theor Applied Genet* 76:815-829

McCouch SR, Chen X, Panaud O, Temnykhn S, Xu Y, Cho YG, Huang N, Ishii T and Blair M (1997) Microsatellite marker development, mapping and applications in rice genetics and breeding. *Plant Mol Biol* 35:89-99

McElroy D, Zhang W and Wu R (1990) Isolation of an efficient actin promoter for use in rice transformation. *Plant Cell* 2:163-171

McKenzie MJ, Mett V, Hugh P, Reynolds S and Jameson PA (1998) Controlled cytokinin production in transgenic tobacco using a copper-inducible promoter. *Plant Physiol* 116:969-977

Medford JI, Horgan R, El-Sawi Z and Klee HJ (1989) Alterations of endogenous cytokinins in transgenic plants using a chimeric *isopentenyl transferase* gene. *Plant Cell* 1:403-413

Mitsuhara I, Ugaki M, Hirochika H, Ohshima M, Murakami T, Gotoh Y, Katayose Y, Nakamura S, Honkura R, Nishimiya S, Ueno K, Mochizuki A, Tanimoto H, Tsugawa Y, Otsuki O and Ohashi Y (1996) Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledomous and monocotyledonous plants. *Plant Cell Physiol* 37:49-59

Mondal WA and Choudhuri MA (1984) Sequential and non-sequential pattern of monocarpic senescence in two rice cultivars. *Physiol Plant* 61:287-292

Morrison RA and Evans DA (1988) Haploid plants from tissue culture: new plant varieties in a shortened time frame. *Bio/Tech* 6:684-690

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473-497

Nagamura Y, Antonio BA and Sasaki T (1997) Rice molecular genetic map using RFLPs and its application. *Plant Mol Biol* 35:89-99

Nam HG (1997) The molecular genetic analysis of leaf senescence. *Curr Opin Biotech* 8:200-207

Nayak P, Basu D, Das S, Basu A, Ghosh D, Ramakrishnan, Ghosh M and Sen SK (1997) Transgenic elite indica rice plants expressing *cryI*Ac-endotoxin of *Bacillus thuringensis* are resistant against yellow stem borer (*Scirpophaga incertulas*). *Proc Natl Acad Sci USA* 94:2111-2116

Negrotto D, Jolley M, Beer S, Wenck AR and Hansen G (2000) The use of phosphomannose-isomerase as a selectable marker to recover transgenic maize plants (*Zea mays* L.) via *Agrobacterium* transformation. *Plant Cell Rep* 19:798-803

Nehra NS, Chibbar RN, Leung N, Caswell K, Mallard L, Baga M and Kartha KK (1994) Self-fertile transgenic wheat plants regenerated from isolated scutellar tissues following microprojectile bombardment with two distinct gene constructs. *The Plant J* 5:285-297

Nguyen KHT, Kane EJ and Dix PJ (1998) Hormonal regulation of senescence in cauliflower (*Brassica olereaca* var *Botrytis*). In: Altman A, Ziv M and Izhar S (eds) Plant Biotechnology and In Vitro Biology in the 21st Century, IX International Congress Plant Tissue Culture. Kluwer Academic Publishers, Dordrect, the Netherlands.

Nizeki H and Oono K (1968) Induction of haploid rice plant from anther culture. *Proc Jpn Acad* 44:544-557

Noh Y-S and Amasino R (1999) Identification of a promoter region responsible for the senescence-specific expression of SAG12. *Plant Mol Bio* 41:181-194

Nooden LD (1988) The phenomenon of senescence and aging. In: Senescence and aging in plants. Nooden LD and Leopold AC (eds), San Diego: Academic Press. pp.1-50

Nooden LD and Leopold AC (1978) Phytohormones and the endogenous regulation of senescence and abscission. In: Phytohormones and Related Compounds: a Comprehensive Treatise. Letham DS (ed), New York: Elsevier/North-Holland Biomedical Press. pp.329-369

Odell J, Caimi P, Sauer B, Russel S (1990) Site-directed recombination in tobacco. *Mol Gen Genet* 223:369-378

Ooms G, Risiott R, Kendall A, Keys A, Lawlor D, Smith S, Turner J and Young A (1991) Phenotypic changes in T-cyt transformed potato plants are consistenet with

enhanced sensitivity of specific cell types to normal regulation by root-derived cytokining. *Plant Mol Bio* 17:727-743

Ori N, Juarez MT, Jackson D, Yamaguchi J, Banowetz GM and Hake S (1999) Leaf senescence is delayed in tobacco plants expressing the maize homeobox gene knotted1 under the control of a senescence-activated promoter. *Plant Cell* 11:1073-1080

Oritani T and Yoshida R (1973) Studies on nitrogen metabolism in crop plants, 12. Cytokinins and abscisic acid-like substances levels in rice and soybean leaves during their growth and senescence. *Proc Crop Science Society Japan* 42(3):280-287

Ow DW, Wood KV, De Luca M, de Wet JR, Helinski DR and Howell SH (1986) Transient and stable expression of the firefly *luciferase* gene in plant cells and transgenic plants. *Science* 234: 856-859.

Park T (1991) Physiological aspects of leaf senescence during spikelet filling in rice (*Oryza sativa* L.) PhD dissertation. University of the Philippines, Los Banos, Laguna.

Park TS, Vergara BS and Alejar AA (1993) Senescence patterns of the upper three leaves of rice (*Oryza sativa* L.) during spikelet filling. *Life Sci* 2(1):29-42

Parker CW and Letham DS (1973) Regulators of cell division in plant tissues. XVI. Metabolism of zeatin by radish cotyledons and hypocotyls. *Planta.* 114:199-218

Pechan T, Jiang B, Steckler D, Ye L, Lin L, Luthe DS and Williams WP (1999) Characterization of three distinct cDNA clones encoding cysteine proteinases from maize (*Zea mays* L.) callus. *Plant Mol Bio* 40 (1): 111-119

Peltonen-Sainio P (1997) Nitrogen fertilizer and foliar application of cytokinin affect spikelet and floret set and surivival in oat. *Field Crops Res* 49:169-176

Peng S, Khush GS and Cassman KG (1994) Evolution of the new plant ideotype. In: Cassman KG (ed) Breaking the yield barrier: Proceedings of a workshop on rice yield potential in favorable environments. International Rice Research Institute, Philippines pp.5-20

Peng S, Cassman KG, Virmani SS, Sheehy J and Khush GS (1999) Yield potentials of tropical rice since the release of IR8 and the challenge of increasing rice yield potential. *Crop Sci* 39:1552-1559

Peng S and Khush GS (2003) Four decades of breeding for varietal improvement of irrigated lowland rice in the International Rice Research Institute. *Plant Production Science* 6(3):157-164

Pinto YM, Kok RA and Baulcombe (1999) Resistance to rice yellow mottle virus (RYMV) in cultivated African rice varieties containing RYMV transgenes. *Nat Biotech* 17:702-707

Porali IK, Marttila SM, Laukkanen K, Herranen M, Ho THD and Mikkonen AA. A GA induced cysteine proteinase EPA expressed in germinated barley grain.Unpublished

Prandhl R and Scoffl F (1996) Heat shock elements are involved in heat shock promoter activation during tobacco seed maturation. *Plant Mol Biol* 31:157-162

Puddephat I (2003) Plant genetic engineering. In: Plant Molecular Breeding. Newbury HJ (ed), Blackwell Publishing Ltd, Oxford, pp. 82-133

Raineri DM, Bottino P, Gordon MP and Nester EW (1990) *Agrobacterium*-mediated transformation of rice (*Oryza sativa* L.). *Bio/Tech* 8:33-38

Rapley R (2002) Molecular analysis and amplification techniques. In: Molecular Biology and Biotechnology. 4th edition, Walker J and Rapley R (eds), The Royal Society of Chemistry, Cambridge, pp.25-66

Ray S and Choudhuri MA (1981) Effects of plant growth regulators on grain filling and yield of rice. *Ann Bot* 47:755-758

Redona ED and MacKill DJ (1996) Molecular mapping of quantitative trait loci in japonica rice. *Genome* 39:395-403

Reddy RM, Ladha JK, Ramos MC, Maillet F, Hernandez RJ, Torrizo LB, Oliva NP, Datta SK and Datta K (1998) Rhizobial lipochitooligosaccharide nodulation factors activate expression of the legume early nodulin gene ENOD12 in rice. *The Plant J* 14:693-702

Reichel C, Mathur J, Eckes P, Langenkemper K and Koncz C (1996) Enhanced green fluorescence by the expression of an *Aequorea* Victoria green fluorescent protein mutant in monocotyledenous- and dicotyledenous plant cells. *Proc Natl Acad Sci* USA 93:5888-5893

Richmond AE and Lang A (1957) Effect of kinetin on protein content and survival of detached *Xanthium* leaves. *Science* 125:650-651

Rood MA (2000) Herbicide resistance: two technologies are on the track for 2001. *Rice J* 103:8-10

Rood MA (2001) Almost here: herbicide-resistant rice varieties expected. *Rice J* 104:16-18

Roslan HA, Salter MG, Wood CD, White MR, Croft KP, Robson F, Coupland G, Doonan J, Laufs P, Tomsett AB, Caddick MX (2001) Characterization of the ethanol-inducible *alc* gene-expression system in *Arabidopsis thaliana*. *Plant J* 28(2):225-35

Rounsley SD, Lin X, Ketchum KA, Crosby ML, Brandon RC, Sykes, SM, Kaul S, Mason TM, Kerlavage AR, Adams MD, Somerville CR and Venter JC Unpublished

Roy M and Wu R (2002) Overexpression of S-adenosylmethionine decarboxylase gene in rice increases polyamine level and enhances sodium chloride-stress tolerance. *Plant Sci* 163:987-992

Rupp H-M, Frank M, Werner T, Strnad M and Schmulling T (1999) Increased steady state mRNA levels of the STM and KNAT1 homeobox genes in cytokinin overproducing *Arabidopsis thaliana* indicate a role for cytokinins in the shoot apical meristem. *The Plant J* 18(5):557-583

Russell JA, Roy MK and Sanford JC (1993a) Physical trauma and tungsten toxicity reduce the efficiency of biolistic transformation. *Plant Physiol* 98:1050-1056.

Russell DR, Wallace K, Martinell BJ and McCabe D (1993b) Stable transformation of *Phaseolus vulgaris* via electric discharge mediated particle acceleration. *The Plant Cell Rep* 12:165-170

Saha S, Nagar PK and Sircar PK (1984) Changes in cytokinin activity during seed germination in rice (*Oryza sativa* L.) during grain filling. *Ann Bot* (London) 54: 1-5

Saha S, Nagar PK and Sircar PK (1986) Cytokinin concentration gradient in the developing grains and upper leaves of rice (*Oryza sativa*) during grain filling. *Can J Bot* 64: 2068-2072

Saijo YS, Hata S, Kyozuka J, Shimamoto K and Izui K (2000) Over-expression of a single Ca^{2+}-dependent kinase confers both cold and salt/drought tolerance on rice plants. *Plant J* 23:319-327

Sankula S, Braverman MP, Jodari F, Linscombe SD and Oard JH (1997) Evaluation of glufosinate on rice (Oryza sativa) transformed with the *bar* gene and red rice (*Oryza sativa*). *Weed Tech* 11:70-75

Sanford JC, Klein TM, Wolf ED and Allen N (1987) Delivery of substances into cells and tissues using a particle bombardment process. *Particulate Science and Tech* 5:27-37

Sano H, Seo S, Orudgev E, Youssefian S, Ishizuka K and Ohashi Y (1994) Expression of the gene for a small GTP binding protein in transgenic tobacco elevates endogenous cytokinin levels, abnormally induces salicylic acid in response to wounding, and increases resistance to tobacco mosaic virus infection. *Proc Natl Acad Sci USA* 91:10556-10560

Schumulling T, Beinsberger S, De Greef J, Schell J and Spena A (1989) Construction of a heat-inducible chimeric gene to increase the cytokinin content in transgenic plant tissue. *FEBS Lett* 249:401-406

Sharma A, Sharma M, Imamura M, Yamakwa M and Machii H (2000) Transgenic expression of cecropin B, an antibacterial peptide from *Bombyx mori*, confers enhanced resistance to bacterial leaf blight in rice. *FEBS Lett* 484:7-11

Sheen J, Hwang S, Niwa Y, Kobayashi H and Galbraith DW (1995) Green-fluorescent protein as a new vital marker in plant cells. *Plant J* 8:777-784

Shimamoto K, Terada R, Izawa T and Fujimoto H (1989) Fertile transgenic rice plants regenerated from transformed protoplasts. *Nature* 388:274-276

Shintani A, Yamauchi D and Minamikawa T (1995) Nucleotide sequence of cDNA for a putative cysteine protease from rice seeds. *Plant Physiol* 107 (3): 1025

Sharma HC, Crouch JH, Sharma KK, Seetharama N and Hash CT (2002) Applications of biotechnology for crop improvement: prospects an constraints. *Plant Sci* 163:381-395.

Sivamani E, Shen P, Opalka N, Beachy RN and Fauquet CM (1996) Selection of large quantities of embryogenic calli from indica rice seeds for production of fertile transgenic plants using biolistic method. *Plant Cell Rep* 15:322-327

Smart CM, Scofield SR, Bevan MW and Dyer TA (1991) Delayed leaf senescence in tobacco plants with *tmr*, a gene for cytokinin production in *Agrobacterium*. *The Plant Cell* 3:647-656

Smigocki, A.C. and L.D. Owens. 1988. Cytokinin gene fused with a strong promoter enhances shoot organogenesis and zeatin levels in transformed plant cells. *Proc Natl Acad Sci USA* 85:5131-5135

Smigocki, AC (1991) Cytokinin content and tissue distribution in plants transformed by a reconstructed *isopentenyl transferase* gene. *Plant Mol Biol* 16:105-115

Smigocki, AC (1995) Expression of a wound-inducible cytokinin biosynthesis gene in transgenic tobacco: correlation of root expression with induction of cytokinin effects. *Plant Sci* 109:153-163

Smigocki, A, Neal JW, McCanna I and Douglas, L (1993) Cytokinin-mediated insect resistance in *Nicotiana* plants transformed with the ipt gene. *Plant Mol Biol* 23:325-335

Snape JW (1989) Double haploid breeding: theoretical basis and practical applications. In: Review of Advances in Plant Biotechnology, 1985-1988: 2nd Int Smp Genetic Manipulation in Crops. Mujeeb-Kazi A and Sitch LA (eds), Mexico and Manila, CIMMYT and IRRI, pp.19-30

Soejima H, Sugiyama T and Ishihara K (1995) Changes in the chlorophyll contents of leaves and in levels of cytokinins in root exudates during ripening of rice cultivars Nipponbare and Akenohoshi. *Plant Cell Physiol* 36(6):1105-1114

Somers DA, Rines HW, Gu W, Kaeppler HF and Bushnell WR (1992) Fertile, transgenic oat plants. *Bio/Tech* 10:1589-1594

Stenesh J (1989) Dictionary of Biochemistry and Molecular Biology. 2nd ed. New York: John Wiley and Sons. pp. 525.

Sudakhar D, Fu X, Stoger E, Williams S, Spence J, Brown DP, Bharati M, Gatehouse JA and Christou P (1998) Expression and immunolocalisation of the snowdrop lectin, GNA in transgenic rice plants. *Transgen Res* 7:371-378

Tada Y, Sakamoto M, Matsuoak M and Fujimura T (1991) Expression of a monocot LHCP promoter in transgenic rice. *The EMBO J* 10(7):1803-1808

Tai TH (2003) Rice biotechnology. In: Rice. Smith CW and Dilday (eds). New Jersey: John Wiley and Sons, Inc. 203-219

Tamaoki M, Kusaba S, Kano-Murakami Y and Matsuoka M (1997) Ectopic expression of a tobacco homeobox gene, *NTH15*, dramatically alters leaf morphology and hormone levels in transgenic tobacco. *Plant Cell Physiol* 38:917-927

Takagi M, Yokota T, Murofushi N, Ota Y and Takahashi N (1985) Fluctuation of endogenous cytokinin contents in rice during its life cycle – quantification of cytokinins by selected ion monitoring using deuterium labeled internal standards. *Agric Biol Chem* 49(11):3271-3277

Tang K, Tinjuangjun P, Xu Y, Sun X, Gatehouse JA, Ronald PC, Qi H, Lu X, Christou P and Kohli A (1999) Particle-bombardment-mediated co-transformation of elite Chinese rice cultivars with genes coferrin resistance to bacterial blight and sap-sucking insect pests. *Planta* 208:552-563

Tang K, Sun X, Hu Q, Wu A, Lin C, Lin H, Twyman RM, Christou P and Feng T (2001) Transgenic rice plants expressing the ferredoxin-like protein (AP1) from sweet pepper show enhanced resistance to *Xanthomonas oryzae* pv *oryzae*. *Plant Sci* 160:1035-1042

Terada R and Shimamoto K (1990) Expression of CaMV35S-GUS gene in transgenic rice plants. *Mol Gen Genet* 220:389-392

Terada R, Nakayama T, Iwabuchii M and Shimamoto K (1993) A wheat histone H3 promoter confers cell division dependent and independent expression of the *gusA* gene in transgenic rice plants. *The Plant J* 3(2):241-252

Thomas H and Howarth CJ (2000) Five ways to stay green. *J of Exp Bot* 51:329-337

Thomas JC, Smigocki, AC and Bohnert HJ (1995) Light-induced expression of *ipt* from *Agrobacterium tumefaciens* results in cytokinin accumulation and osmotic stress symptoms in transgenic tobacco. *Plant Mol Bio* 27:225-235.

Tingay S, McElroy D, Kalla R, Fieg S, Wang M, Thornton S and Brettell R (1997) *Agrobacterium tumefaciens*-mediated barley transformation. *Plant J* 11:1369-1376

Toki S, Takamatsu S, Nojiri C, Ooba S, Anzai H, Iwata M, Christensen AH, Quail PH and Uchimiya H (1992) Expression of a maize ubiquitin promoter-bar chimeric gene in transgenic rice plants. *Plant Physiol* 11:1503-1507

Toriyama K, Arimoto Y, Uchimiya H and Hinata K (1988) Transgenic rice plants after direct gene transfer into protoplasts. *Bio/Tech* 6:1072-1074

Town CD, Haas BJ, Maiti R, Hannick LI, Chan AP, Ronning CM, Smith Jr. RK, Yu CY, Wortman JR, White O and Fraser CM Arabidopsis thaliana chromosome 5 CHR5v07142002 genomic sequence Unpublished

Tran TCH, Hoq ME, Vincent JR, Hodges HK, Hodges T (1999) Controlled excision of a transgene from the rice genome by the Cre/LoxP site-specific recombination. In: Abstracts General Meeting on the International Program on Rice Biotechnology. Phuket, Thailand, p.16

Tsuchiya T, Ohta H, Okawa K, Iwamatsu A, Shimada H, Masuda T and Takamiya K (1999) Cloning of chlorophyllase, the key enzyme in chlorophyll degradation: finding of a lipase motif and the induction by methyl jasmonate. *Proc Natl Acad Sci USA* 96 (26): 15362-15367

Tu J, Zhang G, Datta K, Xu C, He Y, Zhang Q, Khush GS and Datta SK (2000) Field performance of transgenic elite commercial hybrid rice expressing *Bacillus thuringensis* alpha-endotoxin. *Nat Biotech* 18:1101-1104

Turner PC, McLennan AG, Bates AD and White MRH (2000) Instant Notes Molecular Biology. 2nd edition. Oxford, UK: BIOS Scientific Publishers Limited. pp 346.

Twell D, Kelin TM, Fromm ME and McCormick S (1989) Transient expression of chimeric genes delivered into pollen by microprojectile bombardment of regenerable embryogenic bombardment. *Plant Physiol* 91:1270-1274

Twyman RM, Christou P and Stoger E (2002) Genetic transformation of plants and their cells. In: Plant Biotechnology and Transgenic Plants. Oksman-Caldentey KM and Barz WH (eds), New York: Marcel Dekker, Inc. pp. 111-141

Upadhyaya NM, Zhou-ZR, Zhu Q-H, Eamens A, Wang M-B, Waterhouse PM and Dennis ES (2000) Transgenic rice. In: Transgenic Cereals. O'Brien LO and Henry RJ (eds), Minnesota: American Association of Cereal Chemists, Inc. pp. 28-87

Vain P, McMullen MD and Finer JJ (1993) Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. *Plant Cell Rep* 12:83-88

Vain P, De Buyser J, Buitrang V, Haicour R and Henry Y (1995) Foreign gene delivery into monocotyledonous species. *Biotech Adv* 13(4):653-671

Vain P, Worland B, Clarke M, Richard G, Beavis M, Liu H, Kohli A, Leech M, Snape J, Christou P and Atkinson H (1998) Expression of an engineered cysteine proteinase inhibitor (oryzacystatin) for nematode resistance in transgenic rice plants. *Theor Appl Genet* 96:266-271

Vain P, Afolabi AS, Worland B and Snape JW (2003) Transgene behaviour in populations of rice plants transformed using a new dual binary vector system: pGreen/pSoup. *Theo Appl Genet* 107:210-217

Van Loven KV, Beinsberger SEI, Valcke RLM, Van Onckelen HA and Clijsters HMM (1993) Morphometric analysis of the growth of *Phsp70-ipt* transgeic tobacco plant. *J of Exp Bot* 44(268):1671-1678

Van Staden J (1976) The identification of zeatin glucoside from coconut milk. *Physiol Plant* 36:123-126

Van Staden J (1983) Mini review on seeds and cytokinin. *Physiol Plant* 58:340-346.

Van Staden, J, Cook EL and Nooden LD (1988) Cytokinins and senescence. In: Nooden LD and Leopold AC (eds), Senescence and Aging in Plants. London: Academic Press Inc. pp 232-328.

Vasconcelos M, Datta K, Oliva N, Khalekuzzaman M, Torrizo L, Krishnan S, Olivera M, Goto F, Datta SK. 2003. Enhanced iron and zinc accumulation in transgenic rice with the ferritin gene. *Plant Sci* 164:371-378

Vasil V, Castillo AM, Fromm ME and Vasil I (1992) Herbicide resistant fertile wheat plants obtained by microprojectile bomabardment of regenerable embryogenic callus. *Bio/Tech* 10:667-674

Vasil V, Redway F and Vasil I (1990) Regeneration of plants from embryogenic suspension culture protoplasts of wheat (*Triticum aestivum* L.). *Bio/Tech* 8:429-434

Virk PS and Khush GS (2002) Prospects of IRRI's new plant type rice in the tropics.
Wan Y and Lemaux PG (1994) Generation of large numbers of independently transformed fertile barley plants. *Plant Physiol* 104:37-48

Wang YC, Klein TM, Fromm ME, Cao J, Sanford JC and Wu R (1988) Transformation of rice, wheat and soybean by the particle bombardment method. *Plant Mol Bio* 11:433-439

Watanabe H, Abe K, Emori Y, Hosoyama H and Arai S (1991) Molecular cloning and gibberellin-induced expression of multiple cysteine proteinases of rice seeds (oryzains) *J Biol Chem* 266 (25): 16897-16902

Weeks JT, Anderson OD and Blechl AE (1993) Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum*). *Plant Physiol* 102:1077-1084

White, FF (1993) Vectors for gene transfer in higher plants. In: Transgenic Plants, Volume 1, Kung S and Wu R (eds), Academic Press, San Diego, pp.15-48

Wilmink A, Van de Ven BCE and Dons JJM (1992) Expression of the GUS-gene in the monocot tulip after introduction by particle bombardment and *Agrobacterium*. *Plant Cell Rep* 11:76-80

Woo HR, Chung KM, Park J-H, Oh S, Ahn T, Hong SH, Jang SK and Nam HG (2001) ORE9, an F-box protein that regulates leaf senescence in Arabidopsis. *The Plant Cell* 13:1779-1790

Wunn J, Kloti A, Burkardt P, Ghosh Biswas G, Launis K, Iglesias V and Potrykus I (1996) Transgenic indica rice breeding line IR58 expressing a synthetic *cry*IA(b) gene from *Bacillus thuringensis* provides effective insect pest control. *Bio/Tech* 14:171-176

Wuthrich KL, Bovet L, Hunziker PE, Donnison IS and Hortensteiner S (2000) Molecular cloning, functional expression and characterisation of RCC reductase involved in chlorophyll catabolism. *Plant J* 21 (2): 189-198

Yamada T, Ohta H, Shinohara,A, Iwamatsu A, Shimada H, Tsuchiya T, Masuda T and Takamiya K (2000) A cysteine protease from maize isolated in a complex with cystatin. *Plant Cell Physiol* 41 (2): 185-191

Yang J, Wang Z and Zhu Q (1997) Photosynthetic characteristics, dry matter accumulation and its translocation in inter-subspecific hybrid rice. *Acta Agron Sinica* 23:82-88.

Yang J, Peng S, Zhang Z, Wang Z, Visperas RM and Zhu Q (2002a) Grain and dry matter yields and partitioning of assimilates in japonica/indica hybrid rice. *Crop Sci* 42:766-772

Yang J, Zhang J, Zhang Q, Zhu Q and Liu L (2002b) Abscisic acid and cytokinins in the root exudates and leaves and their relationship to senescence and remobilization of carbon reserves in rice subjected to water stress during grain filling. *Planta* 215(4):645-652

Ye X, Babili A, Kloti A, Zhang J, Lucca P, Beyer P and Potrykus I (2000) Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* 287:303-305

Yin Y and Beachy R (1995) The regulatory regions of the rice tungro bacilli form virus promoter and interacting nuclear factor in rice (*Oryza sativa* L.). *Plant J* 7:969-980

Yokoi S, Tsuchiya T, Toriyama K and Hinata K (1997) Tapetum-specific expression of the Osg6B promoter β-glucuronidase gene in transgenic rice. *Plant Cell Rep* 16:363-367

Yoshida S and Cock JH (1971) Growth performance of an improved rice variety in the tropics. *Int Rice Res Newsl* 20:1-15

Yoshida S, Ito M, Nishida I and Watanabe A (2001) Isolation and RNA gel blot analysis of genes that could serve as potential molecular markers for leaf senescence in *Arabidopsis thaliana*. *Plant Cell Physiol* 42 (2): 170-178

Yue S, Yu Z and Yu S (1996) Changes of oxygen-free radical level and endogenous plant hormone content during flag leaf senescence in wheat. *Plant Physiol Comm* 32(5):349-351

Yushibov VM, Il PC, Andrianov VM and Piruzian ES (1991) Phenotypically normal transgenic T-cyt tobacco plants as a model for the investigation of plant gene expression in response to phytohormonal stress. *Plant Mol Bio* 17:825-836

Zhang HM, Yang H, Rech EL, Golds TJ, Davis AS, Mulligan BJ, Cocking EC and Davey MR (1988) Transgenic rice plants produced by electroporation-mediated plasmid uptake into protoplasts. *Plant Cell Rep* 7:379-384

Zhang W, McElroy D and Wu R (1991) Analysis of rice Act5' region activity in transgenic plants. *Plant Cell* 3:1155-1165

Zhang J, Xu R, Elliott M and Chen DF (1997) *Agrobacterium*-mediated transformation of elite indica and japonica rice cultivars. *Molec Biotech* 8(3):223-231

Zhang S, Chen L, Qu R, Marmey P, Beachy R and Fauquet C (1996) Regeneration of fertile transgenic indica (group 1) rice plants following microprojectile transformation of embryogenic suspension culture cells. *Plant Cell Rep* 15:465-469

Zhang R, Zhang X, Wang J, Letham DS, Mckinney SA and Higgins TJV (1995) The effect of auxin on cytokinin levels and metabolism in transgenic tobacco tissue expressing an *ipt* gene. *Planta* 196:84-94

Zhang W and Wu R (1988) Efficient regeneration of transgenic plants from rice protoplasts and correctly regulated expression of the foreign gene in the plants. *Theor Appl Genet* 76:835-840

Zhao C, Johnson BJ, Kositsup B and Beers EP (2000) Exploiting secondary growth in *Arabidopsis*, construction of xylem and bark cDNA libraries and cloning of three xylem endopeptidases. *Plant Physiol* 123 (3): 1185-1196

Zheng K, Subudhi PK, Domingo J, Magpantay G and Huang N (1995) Rapid DNA isolation for marker assisted selection in rice breeding. *Rice Genetics Newsl* 12: 255–258.

Zhu Q, Zhang Z, Yang J and Wang Z (1997) Source-sink characteristics related with the yield of inter-subspecific hybrid rice. *Sci Agric Sinica* 30:52-59

Zuo J and Chua NH (2000) Chemical-inducible systems for regulated expression of plant genes. *Curr Opin Biotech* 11:146-151

Zuo JR, Niu QR, Moller SJ and Chua NH (2001) Chemical-regulated, site-specific DNA excision in transgenic plants. *Nat Biotech* 19:157-161

Zupan J, Muth TR, Draper O and Zambryski P (2000) The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. *Plant J* 23:11-28

APPENDIX

Murashige and Skoog (MS) medium

Component	Concentration (mg/litre)
Inorganic salts	
NH_4NO_3	1,650.000
KNO_3	1,900.000
$CaCl.2H_2O$	8,000.000
$MgSO4.7H_2O$	370.000
KH_2PO_4	170.000
KI	0.930
H_3BO_3	6.200
$MnSO_4.4H_2O$	16.100
$ZnSO_4.7H_2O$	8.600
$Na_2MoO_4.2H_2O$	0.250
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
$FeSO_4.7H_2O$	27.850
$Na_2EDTA.2H_2O$	37.250
Vitamins	
Myo-inositol	100.000
Nicotinic acid	0.500
Pyridoxine-HCL	0.500
Thiamine-HCL	0.100
2,4-D	2.000
Sugars	
Sucrose	30,000
Agar	8,000

Adjust the pH 5.8 before autoclaving.

Lauria-Bertani (LB) medium

Component	Concentration (g/litre)
Bacto-tryptone	10
Bacto-yeast extract	5
NaCl	5

Adjust the pH 7.2-7.5 before autoclaving.

GUS staining solution

Components	mg/100 ml
X-gluc	25
NaH_2PO_4	1,380
Triton X-100	0.05%
Methanol	2%
pH	7.0